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PRINCIPAL INVESTIGATOR: Stephen D. Williams, M.D.

CONTRACTING ORGANIZATION: Indiana University  
Indianapolis, IN 46202-5167

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### INTRODUCTION

The objective of this proposal is to develop gene therapy strategies that inhibit ovarian cancer cell growth and selectively improve sensitivity of ovarian cancer cells to chemotherapy and radiation. The proposal focuses on *in vitro* and *in vivo* "proof-of-concept" studies of target discovery.

Four highly interactive projects make up this proposal. We have identified key genes that may be effective targets in ovarian cancer therapy. The first three projects seek to identify alterations in these genes which, either alone or in combination with chemotherapy or radiation, will efficiently kill ovarian cancer cells. Project 4 will identify promoters that allow for high expression of our key gene(s) in ovarian cancer cells but minimal expression in normal tissues.

To increase sensitivity of ovarian cell lines to chemotherapy and radiation by genetic modification, Projects 1 and 2 will modify DNA repair pathways, and the most promising gene targets identified are being tested in tumor xenografts. Project 3 investigates the role of the cyclin dependent kinase inhibitors (CDKI's) in ovarian cancer cell cycle control and tests the hypothesis that sustained overexpression of p27<sup>kip1</sup> and p53<sup>kip2</sup> will suppress growth and/or cause programmed cell death in ovarian cancer cell lines. The effect of adding chemotherapy will be studied, and the most promising strategies from Project 3 will then be evaluated in xenografts by our animal core. In Project 4, human cDNA microarray technology is being used to identify ovarian cancer specific promoters that will allow effective expression of these altered genes in ovarian tumor cells but limited expression in normal cells.

**Project 1: Therapeutic manipulation of the DNA base excision repair pathway for ovarian tumor sensitization (PI: Mark R. Kelley, Ph.D.)**

**Task 1.** Identify altered Ape1 dominant-negative proteins that sensitize ovarian tumor cell lines to chemo-/IR therapy (months 1-24). Mutants of Ape1, which bind substrate DNA with wild-type or better affinity but do not execute repair, will be expressed in ovarian (HeyC2 and SKOV-3X) cell lines using the ecdysone-inducible system (months 1-36). End points such as cell growth, cytotoxicity, DNA damage, and apoptosis will be assessed. Treatments to be used to monitor for improved cytotoxicity include methyl methanesulfonate (MMS), mafosfamide (clinical agent), and ionizing radiation (IR). (months 1-36)

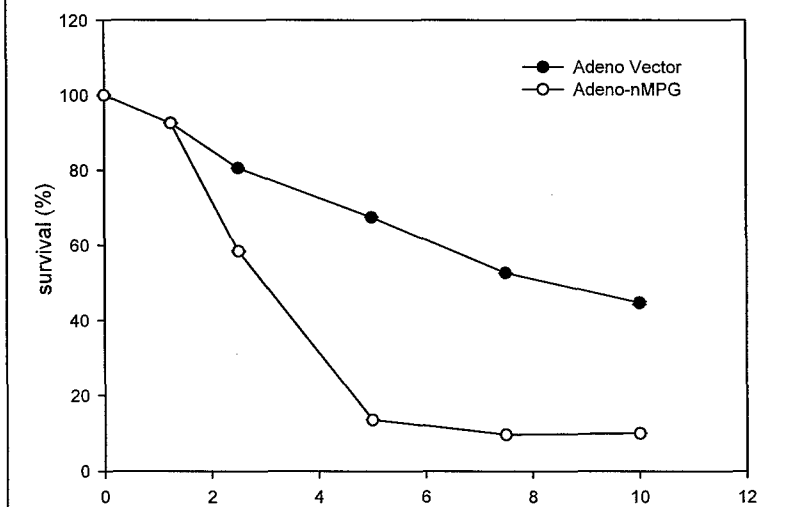
No new data since last report. The studies from last year and presented in previous reports have been verified and repeated numerous times using Ape1 siRNA to enhance cell killing to various DNA damaging agents.

**Task 2.** Determine the effectiveness of MPG and MPG<sup>mutants</sup> overexpression at killing ovarian tumor cells following alkylating agent chemotherapy. We will make site directed mutations in the active site of MPG which should allow for binding to DNA lesions without removing damaged base. (months 1-12). The MPGs will be overexpressed in ovarian cell lines, and survival will be monitored following exposure to MMS, mafosfamide and cisplatin. (months 1-36)

No new data since last report. We have now confirmed that overexpressing both nuclear or mitochondrial targeted MPG leads to ovarian cancer cell killing with the alkylating agent temozolomide (TMZ). The addition of MPG to IGROV ovarian cancer cells results in almost complete killing at 5 mM TMZ dose compared to 50% survival of cells treated with TMZ alone at 10 mM. This is roughly a **10-fold increase** in the LD50 for TMZ. This is clinically relevant as TMZ is slated for phase II ovarian clinical trials and enhancing its effect could be an exciting and important therapeutic treatment for ovarian cancer. Similar results with MMS are also observed, as well as with the mitochondrially targeted MPG. We have also shown that the method of cell death is through single and double-stranded DNA breaks and apoptosis.

Figure 2.1 Nuclear targeted MPG dramatically enhances the killing effectiveness of TMZ.

TMZ and MPG treatment of Igrov p53 cells (MTT assay)



**Task 3. Determine the effects of co-overexpression of Ape1<sup>mutants</sup>, MPG and MPG<sup>mutants</sup>. We will monitor whether combined expression enhances the ovarian tumor cell killing effect of lower doses of chemo-/IR agents administered alone or simultaneously. (months 12-36). Combined expression will be accomplished using either two independent expression plasmids or IRES (internal ribosome entry site) elements and the same expression construct, as well as an adenoviral expression vector. (months 12-36)**

Same as last report. Given the results in Task 1 and Task 2, this does not appear to be a viable option at this time and the main focus is on the nuclear and mitoMPG constructs along with Ape1 small molecule inhibitors together which, effectively, is similar to using Ape1 mutants with MPG, but much easier to co-treat.

**Task 4. Determine *in vivo* chemo- and IR-sensitivity of ovarian cells expressing Ape1<sup>mutants</sup>, MPG, or MPG<sup>mutants</sup>. (months 6-36). Ovarian cell lines carrying mutant Ape1, MPG, or MPG<sup>mutants</sup> genes will be used to produce tumors in the Xenograft Core A. (months 6-36). Dose-response studies will be performed on tumors produced from non-transfected and transfected cells to determine if the addition of the Ape1 mutants or MPG/MPG<sup>mutant</sup> gene product increases sensitivity to the drugs. (months 6-36). Sensitivity to mafosfamide, cisplatin, and IR will be tested. (months 6-36)**

No new progress on these studies to report at this time. These studies are still in progress. They were delayed due to changes in the constructs chosen as well as requiring new IBC approval for the adenoviral and animal studies.

Related Publication:

Tell, G., Damante, G., Caldwell, D. and Kelley, M.R. (2005) The intracellular localization of APE1/Ref-1: More than a passive phenomenon? *Antioxidants and Redox Signaling* 7: 365-382.

**Project 2: Targeted inhibition of a key DNA repair enzyme, DNA-dependent protein kinase, in ovarian cancer co-therapy (PI: S-H. Lee, Ph.D.)**

**Task 1. To develop a peptide that specifically inhibits DNA-PK kinase activity by interfering with the interaction of DNA-PK catalytic subunit (DNA-PKcs) and Ku70/Ku80**

**1.1. Screening for candidate peptides that interact with N-terminal domains of Ku70-Ku80**

Studies from us and others indicated that DNA-PKcs interacting domain is localized at the extreme C-terminus of Ku80 (amino acids 720-732) (Gell and Jackson, 1999; Kim & Lee, unpublished). Since the C-terminus of Ku80 is also likely involved in heterodimer assembly and DNA termini binding, this region (amino acids 720-732 of Ku80) was selected to synthesize a target peptide that would prevent DNA-PKcs from binding to Ku70/Ku80 regulatory subunits. To deliver a peptide to the cancer cells, a cell-permeable peptide import domain and the nuclear localization domain were added to the target peptide to obviate the need for permeabilization or microinjection of individual cells (Kim *et al*, 2002).

We selected a target peptide representing amino acids 720-732 of Ku80. This domain not only interacts with DNA-PKcs, but may also be involved in Ku heterodimer assembly and DNA termini binding. Peptide-based inhibitor also contains a hydrophobic signal peptide, so-called membrane-translocating carrier, which not only facilitates secretion of protein, but also is important for importing synthetic peptide into cell (Lin YZ *et al*, 1995). We therefore synthesized a 38-residue peptide (HNI-38) comprising the signal peptide sequence (AAVALLPAVLLALLAP), nuclear localization signal NLS (VQRKRQKLM), followed by a tyrosine (Y) residue, and 12-residue of peptide inhibitor sequence (EGGDVDDLLDMI) representing the C-terminus of Ku80 (amino acids #721-732).

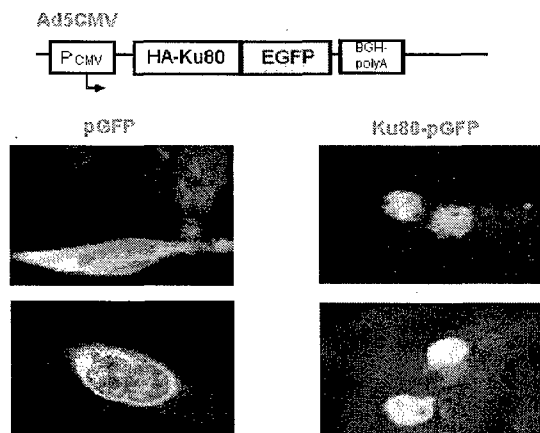
**1.2. Effect of target peptide on the functions of DNA-PK and/or Ku70/Ku80**

The target peptide (HNI-38) inhibited the interaction of DNA-PKcs with Ku70/Ku80 and the binding of Ku complex to duplex DNA. DNA-PKcs and Ku70/Ku80 are abundant proteins approximately  $5 \times 10^5$  molecules per human cells (Lee and Kim, 2002) and most of Ku70/Ku80 heterodimer exists in cell extracts without forming a complex with DNA-PKcs in the absence of DNA (Hammarsten and Chu, 1998). Target peptide (HNI-38) was examined for its effect on the interaction between DNA-PKcs and Ku70/Ku80 in the presence of dsDNA. Although it was marginal, the addition of increasing amount of target peptide (HNI-38) not the control peptide (HN-26) led to a decrease in DNA-PKcs associated with dsDNA (Kim *et al*, 2002). We also carried out a dsDNA pulldown assay to examine the effect of HNI-38 on Ku's DNA binding activity. Target peptide (HNI-38) significantly interfered with binding of Ku complex to dsDNA under the conditions where control peptide (HN-26) showed virtually no effect (Kim *et al*, 2002). This result suggests that target peptide not only affects the interaction between DNA-PKcs and Ku, but also interferes with the Ku's DNA binding activity. Efficacy of target peptide was also analyzed for DNA-PK kinase activity *in vitro*. DNA-PK kinase activity was inhibited up to 50% in the presence of target peptide under the condition where a control peptide showed minimal effect (Kim *et al*, 2002), which strongly supported a notion that target peptide binds to DNA-PKcs and interferes with the interaction between DNA-PKcs and the Ku complex.

**Task 2. To determine if a peptide-based inhibitor of DNA-PK (or negative-dominant mutant of Ku80) lowers anti-cancer drug resistance and facilitate killing of ovarian cancer cells**

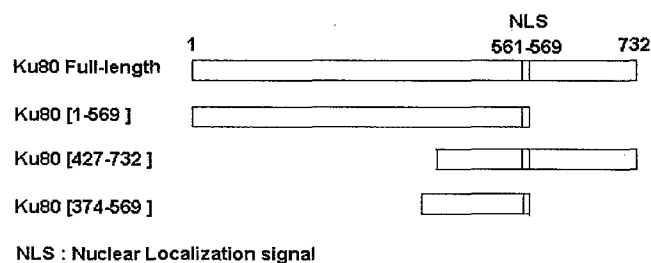
## 2.1. *In vivo* expression system

To deliver a specific inhibitor (or mutant protein) to the nucleus of ovarian cancer cells, Ku80 containing EGFP at the C-terminus was cloned into an adenoviral vector (Ad5CMV) expression system (Figure 1). A control containing only GFP showed ubiquitous expression, while Ku80 was localized in the nucleus. This adenoviral vector will be used to express a negative-dominant mutant of Ku80 that can interfere with the *in vivo* function of Ku80.



**Figure 1.** Expression of GFP (left panels) or hemagglutinin (HA)-tagged Ku80-pGFP (right panels) in ovarian cancer cells (Hey).

A series of Ku80 deletion mutants are being generated and will be tested for their negative dominant phenotype in inhibiting cancer cell growth after cloning them into adenoviral vector (Ad5CMV vector; Figure 2, below). Once prepared, these mutants will be examined for their effect on cancer cell growth.



## 2.2. Effect of a peptide-based inhibitor in chemotherapy drug or IR therapy in ovarian cancer cells

Cells lacking DNA-PK catalytic subunit showed increased sensitivity to DNA damaging drugs or IR (Lees-Miller et al., 1995; Kirchgessner et al., 1995), suggesting that DNA-PK activity is essential for DNA repair and cell survival upon DNA damage. We therefore tested whether a targeted inhibition of DNA-PK by a peptide HNI-38 would sensitize ovarian cancer cells upon treatment of ionizing radiation or chemotherapeutic drug (cisplatin). Two ovarian cancer cells (Hey and Hey-C2) were treated with either control (HI-26) or target peptide (HNI-38) and tested for the efficacy of DNA-PK inhibitory peptide on lowering resistance of cells in response to ionizing radiation using standard colony count cell survival assay. Both control and target peptides did not show any effect on cell growth in the absence of ionizing radiation. However, cells treated with IR showed significant cell growth inhibition in the presence of target peptide but not with control peptide (Kim *et al*, 2002), suggesting that cell



growth inhibition by target peptide occurs through targeting DNA-PK activity. Cells treated with cisplatin, although not as effective as those treated with IR, also showed inhibitory effect on cell growth in the presence of HNI-38 (Kim *et al*, 2002).

**2.3. *In vivo* efficacy of chemotherapy drug (doxorubicin or cisplatin) or IR therapy on shrinkage of mouse xenograft tumor** The xenograft model is an *in vivo* tool to examine the efficacy of a mutant Ku80 in drug resistance of ovarian cancers. Once we select Ku80-mutant from the cell survival study, the mutant will be tested in shrinkage of mouse xenograft tumor. A drug-resistant ovarian cancer cells (SKOV3X) will be transfected with the pVgRXR plasmid and the pIND containing a mutant Ku80 gene. Transfected cells will be injected at two subcutaneous sites on athymic mice ( $1.0 \times 10^7$  cells/ mouse). After a suitable time for establishment of tumors, the animals will be treated with chemotherapeutic drug (adriamycin or cisplatin) and the resultant tumor mass will be assessed during an additional growth period. The subcutaneous site will serve as a convenient, visible site for monitoring of tumor growth. The mouse will also receive an intraperitoneal injection of cells ( $1.0 \times 10^6$  cells/mouse) to form orthotopic tumors. Throughout the experimental treatment period, the volume of the subcutaneous tumor will be estimated by measuring its length, breadth and depth with a set of calipers. Animals will be treated with drug twice per week for 3 weeks and tumor growth will be followed for another 3 weeks. At the end of the experiment, tumor (both subcutaneous and intraperitoneal) will be excised and weighed.

**NOD/SCID mouse ovarian cancer model:**

The system we will use is a human ovarian cancer in the NOD/SCID mouse xenograft model. Six-to-eight-week-old NOD/SCID mice will be anesthetized with methoxyflurane. Following anesthesia, a small subcutaneous incision will be made in the abdomen, and  $10^6$  ovarian cancer cells will be injected. Subsequent studies after the baseline assessments of implants of SKOV3X cells have been made will involve implantation of cells resulting from Aim 3 and evaluation of tumors developing in mice. *In vivo* correlation will provide valuable information regarding the effects on the intact animal of the specific modifications in the ovarian cancer cells. Thus, we should be able to determine the effects of a mutant Ku80 on the growth and progression of ovarian cancers *in vivo*. This obviously leads to the potential for translation of these findings to women with ovarian cancer.

***In vivo* tumor volume assessment: The positron emission tomography (PET)**

Imaging analysis has obvious advantage over direct tumor volume measurement since it can monitor tumor shrinkage over time without sacrificing mice. FdG or  $^{11}\text{C}$ -choline will be used as a tracer for tumor in imaging analysis under guidance of Dr. Gary Hutchins (Director, PET Imaging Facility, IUSM).

In summation, DNA-PK/Ku70-80 complex is essential for DNA repair as well as cell cycle arrest in response to DNA damage, which contributes to cell survival by protecting cells from apoptosis. Cells treated with target peptide not control peptide showed a noticeable decrease in dsb repair following high dose of IR, suggesting that HNI-38 specifically targets DNA-PK *in vivo* and interferes with dsb repair activity through inhibition of DNA-PK activity. Targeted inhibition of DNA-PK by HNI-38 also caused cell growth inhibition only when cells were treated with IR, suggesting that HNI-38 targeted DNA-PK and lowered resistance of cells in response to IR, which eventually causes growth inhibition of both ovarian cancer cells. Treatment of cells with HNI-38 also showed additive effect on cell growth inhibition in response to cisplatin treatment. This observation is in keeping with previous findings that DNA-PK is directly involved in NER action in mammals (Muller *et al.*, 1998). It also supports a notion that a targeted inhibition of DNA-PK would sensitize cancer cells upon treatment of chemotherapeutic drugs such as cisplatin. Together, our study described here not only validates DNA-PK as a useful

molecular target for the treatment of drug-resistant cancer cells, but also supports physiologic role for DNA-PK in IR or chemotherapy drug resistance of cancer cells.

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**Project 3: Cyclin dependent kinase inhibitors as targets in ovarian cancer.  
(PI: Maureen Harrington, Ph.D.)**

**Task 1: Mutations will be generated in the *KIP2* gene at amino acid residues in common with p27<sup>Kip1</sup> protein that are known to control p27<sup>Kip1</sup> protein stability. Using the p57<sup>Kip2</sup> mutants, we will determine if the mutations affect p57<sup>Kip2</sup> protein stability, subcellular location and/or function in normal and in tumor derived ovarian epithelial cell lines.**

- **Site-directed mutagenesis of the p57<sup>Kip2</sup> gene, subcloning of the cDNAs encoding wild-type and mutant *KIP2* into mammalian expression vectors (months 0-6).**

This step was completed during year 1; the p57<sup>Kip2</sup> mutant was generated and cloned into pcDNA3.1(-)/Myc-His (Invitrogen Corp., San Diego, CA), a mammalian expression vector.

- **Introduction of mammalian expression vectors into non-tumorigenic immortalized ovarian surface epithelial cell line (IOSE) and into epithelial ovarian cancer cell lines (HeyC2 and SKOV3X) (months 6-12).**

This step was completed during year 1; the vectors have successfully expressed wild-type and mutant p27<sup>Kip1</sup> and p57<sup>Kip2</sup> proteins.

- **Comparison of wild-type and mutant p57<sup>Kip2</sup> protein activity, stability and subcellular location by western blotting and CDK2 kinase assays (months 6-12).**

During year 1 we determined that the subcellular location of the wild-type and mutant p57<sup>Kip2</sup> was nuclear. We also found that the mutated p57<sup>Kip2</sup> (T342A) does not stabilize the protein.

During year 2 we determined that CDK2 coimmunoprecipitated with both the wild-type and mutant p57<sup>Kip2</sup> proteins. However, a greater amount of CDK2 was associated with the wild-type p57<sup>Kip2</sup> when compared to the level of CDK2 coimmunoprecipitated with the mutant p57<sup>Kip2</sup>.

**Conclusions:** Based upon the studies completed in years 1 and 2 we concluded that p57<sup>Kip2T342A</sup> and wild-type p57<sup>Kip2</sup> have similar activities, thus the remaining studies focused only on p27.

**Task 2: To determine if transduction of stabilized forms of p27<sup>Kip1</sup> and/or mutated p57<sup>Kip2</sup> inhibit the growth of epithelial ovarian cancer cell lines grown *in vitro* and as xenografts in nude mice.**

- **Subcloning of cDNAs encoding wild-type p27<sup>Kip1</sup> and p27<sup>Kip1/T187A</sup> (encodes the stabilized p27<sup>Kip1</sup> protein) into inducible mammalian expression vectors. Generation of stable epithelial ovarian cancer cell lines (HeyC2 and SKOV3X) containing these cDNA under the control of an ecdysone-inducible promoter (months 12-15).**

The results from the studies conducted during year 1 indicated that we could not use an inducible system to express the p27<sup>Kip1</sup> protein in cells. Therefore during year 2, we used folate to target expression vectors to the folate receptors on ovarian cancer cells.

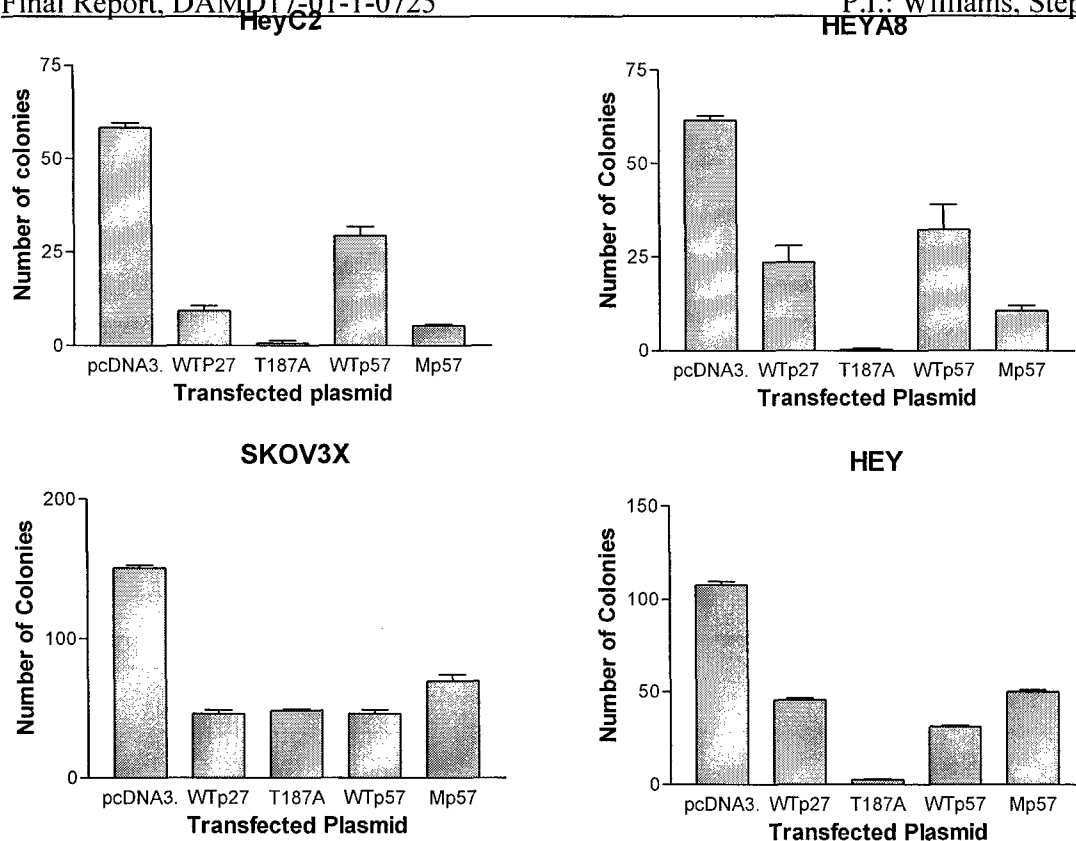
Results of these studies revealed that folate-PEG-poly-L-lysine was able to transfect both the SKOV3X and HeyC2 cell lines (Table 1). It should be noted that the HeyC2 cells showed a higher level of luciferase expression when transfected by the folate-PEG-poly-L-lysine than when transfected with the commercial liposomal reagent, Fugene 6. Thus, the folate receptor may be a useful target for tumor-specific drug delivery. We are currently studying folate conjugated to liposomes as further advancement for the delivery of plasmid DNA to ovarian cancer tumor cells.

**TABLE 1: LUCIFERASE REPORTER GENE EXPRESSION AFTER TRANSFECTION BY FOLATE-PEG-POLY-L-LYSINE COMPLEX**

<u>SKOV3X Cells</u>	<u>Relative Light Units</u>
pEGFPluc only	127
pEGFPluc: folate/PEG/poly-lysine	76,489
pEGFPluc: Fugene 6	399,580
<u>HeyC2 Cells</u>	<u>Relative Light Units</u>
pEGFPluc only	141
pEGFPluc: folate/PEG/poly-lysine	82,425
pEGFPluc: Fugene 6	8,592

- **Growth assays will be performed to determine if cell growth is inhibited and apoptosis is induced in an ecdysone regulated manner (months 15-24).**

As mentioned above, an ecdysone regulated system is not feasible for our studies. In year 2 colony assays were used as an alternative method to determine if the expression of CDKIs will inhibit the growth of epithelial ovarian cancer cells. Results of these assays show that the most efficient and consistent inhibitor of tumor cell growth is p27<sup>Kip1/T187A</sup> (Fig. 2).



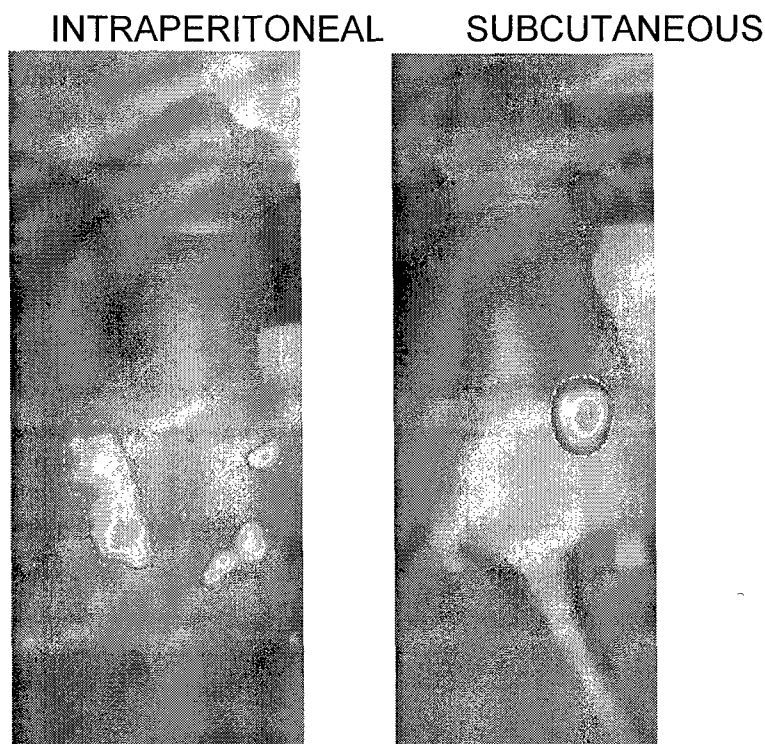
**FIGURE 2: COLONY ASSAYS SHOWING THE INHIBITION OF TUMOR CELL GROWTH BY WILD-TYPE AND MUTANT p27 AND p57.**

• *In vivo* growth assays using xenograft nude mouse model. Cell lines expressing wild-type and mutant *KIP1*, and potentially *KIP2* in an ecdysone regulated manner will be introduced into animals. We will determine if the growth of the introduced tumor cells is modulated in an ecdysone regulated manner. These studies will be performed by Core A.

Because we have determined that the ecdysone system is not suitable for this study, we are determining whether the folate-receptor targeting strategy can be used to target the expression of p27<sup>Kip1</sup> protein to ovarian cancer cells *in vivo*.

During year 2 of this grant we started to develop an *in vivo* bioluminescence method to detect ovarian tumors in the nude mouse model. This has been done in collaboration with Core A. Briefly, the HeyC2 cell line has been stably transfected with pGL3 (Promega, Madison, WI), a luciferase reporter vector, creating the cell line termed HeyC2-luc. Female nude mice were inoculated with either  $5.5 \times 10^6$  HeyC2-luc cells subcutaneously (s.c.) or intraperitoneally (i.p.) with  $3.0 \times 10^5$  HeyC2-luc cells. Nine days after tumor implantation, the mice were anesthetized and given an i.p. injection of luciferin (the substrate for luciferase). The animals were then imaged with a NIGHTOWL Molecular Light Imager (Berthold Technologies, Oak Ridge, TN). This imaging system can detect photons of light emitted from the luciferase-luciferin enzymatic reaction within the tumor cells implanted in the mice. Because photons of light can pass through the skin and other tissues of the animal, the mice do not require any surgery to visualize tumors and can be imaged many times during the course of tumor development. Results from animals imaged nine

days post tumor implantation are shown (Fig. 3). Most significant is the fact that the intraperitoneal tumors were readily detectable by *in vivo* bioluminescence, yet they were undetectable by standard methods of external gross examination and palpation.



**FIGURE 3: IN VIVO BIOLUMINESCENCE IMAGING OF NUDE MICE IMPLANTED WITH LUCIFERASE-EXPRESSING TUMOR CELLS**

**Task 3:** To determine if treatment of cells that express stabilized forms of p27<sup>Kip1</sup> and/or mutated p57<sup>Kip2</sup> with taxanes and /or platins increase epithelial ovarian cancer cell death *in vitro* and in cells grown as xenografts in nude mice.

- Cell growth assays will be performed with cells stably expressing wild-type and/or mutant *KIP1* and/or *KIP2* in an ecdysone regulated manner, in the presence of chemotherapeutic agents studied in Projects 1 and 2 as well as the taxanes and platins to be studied in this project to determine if a higher degree of cell death is achieved (months 24-36).
- Growth assays using xenograft nude mouse model. Cell lines expressing wild-type and mutant *KIP1*, and potentially *KIP2* in an ecdysone regulated manner will be introduced into animals. We will determine if death of the introduced tumor cells is enhanced when chemotherapeutic agents are administered during the induction of wild-type and/or mutant *KIP1* and/or *KIP2*. These studies will be performed by Core A (months 24-36).

The studies described in Task 3 have primarily focused on developing our research strategy that involves following the growth of ovarian epithelial cells *in vivo* that have been engineered to express firefly luciferase. This year we were able to determine that we could follow the growth of an ovarian epithelial cell line *in vivo* overtime following intraperitoneal injection (Figure 4) and subcutaneous injection (Figure 5).

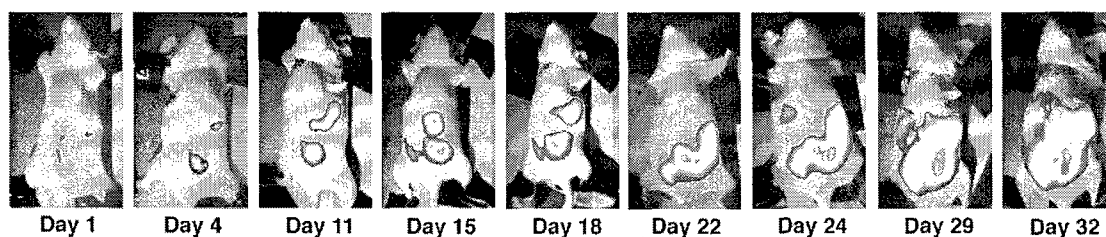


Figure 4: Growth of an ovarian epithelial cell line following intraperitoneal injections. The development of intraperitoneal tumors can be followed even at very early time points using *in vivo* bioluminescence imaging. A nude mouse was injected i.p. with  $3 \times 10^5$  HEYC2luc cells. The next day the mouse was imaged using the NightOWL system as described earlier. Further images were obtained at various time points until the animal displayed significant abdominal distension.

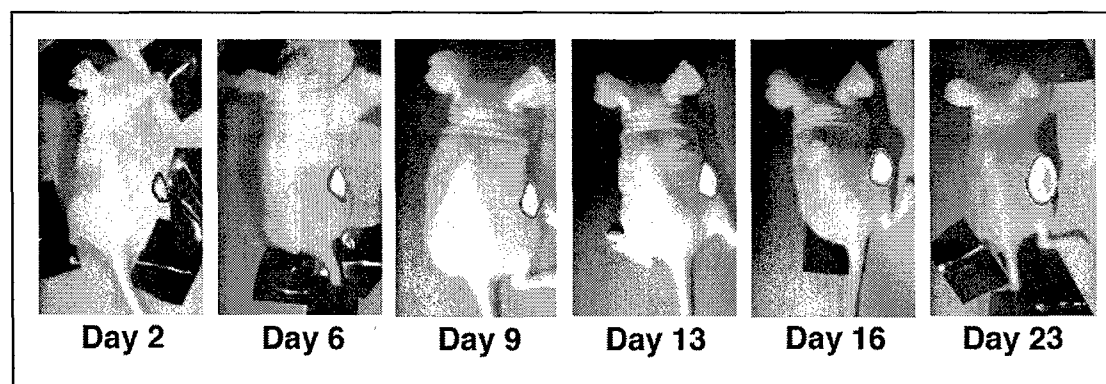


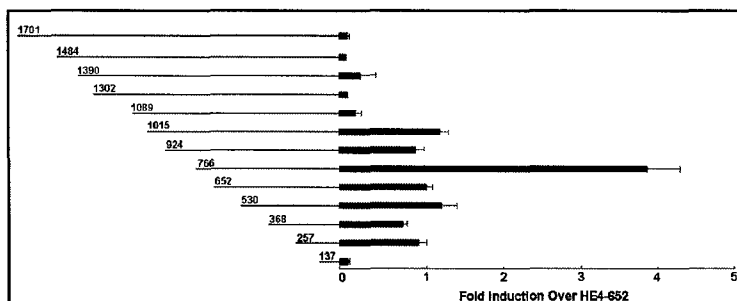
Figure 5. Time dependent growth of an ovarian epithelial cell line following subcutaneous injection. *In vivo* bioluminescence imaging using the NightOWL system. A nude mouse was injected subcutaneously (s.c.) in the hind flank with  $5 \times 10^6$  HEYC2luc cells (luciferase expressing ovarian cancer cell). At various time points after the tumor implantation, luciferin was administered intraperitoneally (i.p.). The mouse was placed in the NightOWL system acquisition chamber. A series of 20 images, each with a 2-minute integration time, was collected. A pseudocolor image representing light intensity was superimposed over a gray scale reference image. The images shown here are at the peak signal intensity for each time point. Tumor growth can be visualized over time.

**PROJECT 4**  
**Identification of Ovarian Tumor-Specific Promoters**  
**(PI: Ken Nephew, Ph.D.)**

The goal of Project 4 is to design a promoter that will generate high levels of expression of the therapy-sensitizing genes identified by Projects 1-3 in ovarian cancer cells but minimal expression in other critical tissues. The development of an ovarian-tumor specific promoter is crucial for using gene therapy to treat ovarian cancer, and Project 4 is geared towards generating a therapeutic vector for the disease.

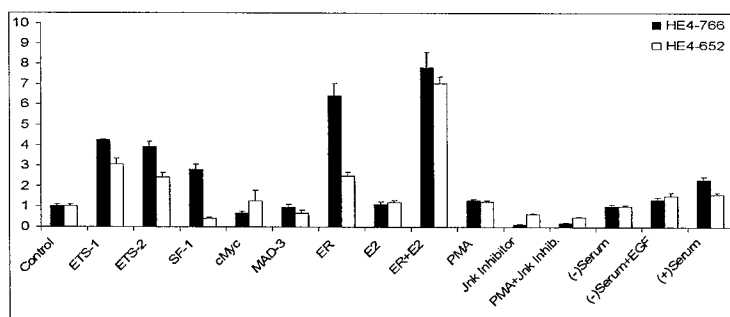
**Task 1 Complete analysis of full length HE4 promoter**

Having discovered an ovarian cancer specific promoter (1) (*see attached manuscript*), we set out to determine if the upstream promoter region contained enhancer elements or other transcription factor binding sites that would allow for increased transcriptional activity of the HE4 promoter without altering its ability to selectively target ovarian cancer cells. We systematically created 13 deletion constructs of the full length HE4 promoter (-1701 to -137 relative to HE4 ATG start site). Included in this group is the original pHE4-652 (1). Each promoter fragment was cloned upstream of the pGL3-basic vector (Promega). The 13 deletion constructs were transiently transfected into A2780 human ovarian cancer cells, and their respective activities were compared to pHE4-652. Constructs surrounding the pHE4-652 construct had similar or less activity than that of 652, with the exception of the pHE4-766 construct, which displayed a four-fold increase in activity compared to pHE4-652 (**Figure 1**). pHE4-766 contained an additional 114bp upstream of the pHE4-652 construct. In order to better understand why the pHE4-766 appeared to be more active than pHE4-652, we examined the transcription factor binding sites of the two HE4 candidate promoters. Binding sites for putative factors likely responsible for high HE4 activity in ovarian cancer as well as transcriptional repressors that might confer its specificity were determined using the promoter prediction software MatInspector ([www.genomatix.de](http://www.genomatix.de)). A total of 203 transcription factor motif families within the 766bp promoter region were found, with 30 families unique to the region between 766 and 652. Among the unique motif families contained within the 652 to 766 region were TCF/LEF, C-Abl, myb, and nur binding sites. The ability of these coactivator or corepressor factors to regulate HE4 promoter activity was analyzed using cotransfection of the expression plasmids along with HE4-luciferase construct in A2780 cells (**Figure 2**). We are currently examining these factors for their contribution to pHE4-766 transcriptional activity.



**Figure 1. Promoter activity of serial deletion constructs of the HE4 promoter in A2780 cells.** A2780 human ovarian cancer cells were transiently transfected with sequentially deleted luciferase reporter fusion-gene constructs. The promoter activity of each construct is expressed as a fold induction relative to the 652bp construct.

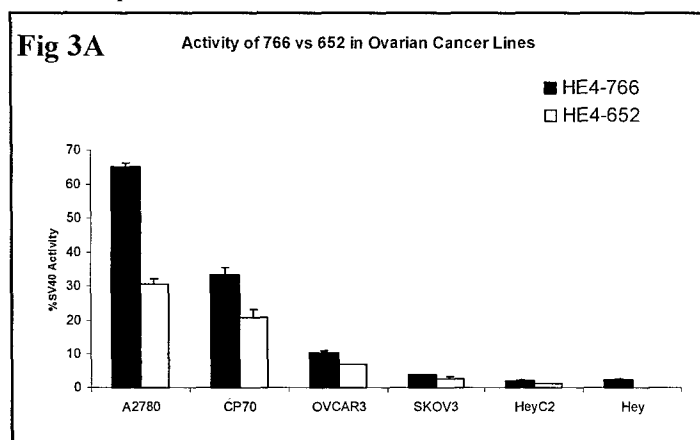


**Task 2 Analysis of optimal construct in ovarian cancer cell lines and non ovarian cell lines**

**Figure 2. Transcription factor analysis in A2780 cells.** A2780 human ovarian cancer cells were cotransfected with 750ng pHE4-652 or pHE4-766 along with 250ng transcription factor expression plasmids for 5 hours followed by media change and incubation for 24 hrs. Drugs were added after 5 hour transfection. E2; 17 $\beta$ -estradiol (10nM), PMA; Phorbol-12-myristate 13-acetate (50nM), EGF; epidermal growth factor (10nM), JNK; SP600125 (60 $\mu$ M).

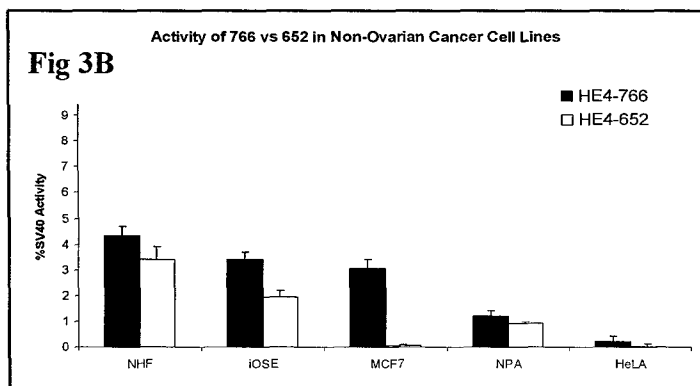
The ETS family of transcription factors appears to increase activity in both pHE4-652 and 766. The SF-1 transcription factor, though present in both promoters, appears to selectively activate pHE4-766. The estrogen receptor also enhances transcriptional activity of pHE4-766, and with the addition of 17 $\beta$ -estradiol, pHE4-652 activity is also enhanced, suggesting that ER can enhance basal transcription of the longer construct, but 17 $\beta$ -estradiol may be needed to stabilize ER binding in the shorter 652 construct. PMA, a potent activator of AP1 sites, does not appear to activate either promoter construct. In addition, inhibition of JNK activity also appears to not alter promoter activity.

To determine the extent of pHE4-766 activity in ovarian cancer cell lines, we performed transient transfection assays in a panel of epithelial ovarian cancer cells. pHE4-766 was active in 3 of 6 ovarian cancer cell lines. pHE4-766 also displayed a higher activity in each of these cell lines compared to that of pHE4-652 (**Figure 3A**). To determine promoter specificity, pHE4-652 and pHE4-766 were also examined in several non ovarian cancer cell lines. Both pHE4-652 and pHE4-766 remained inactive in the non-ovarian cancer cell lines (**Figure 3B**).



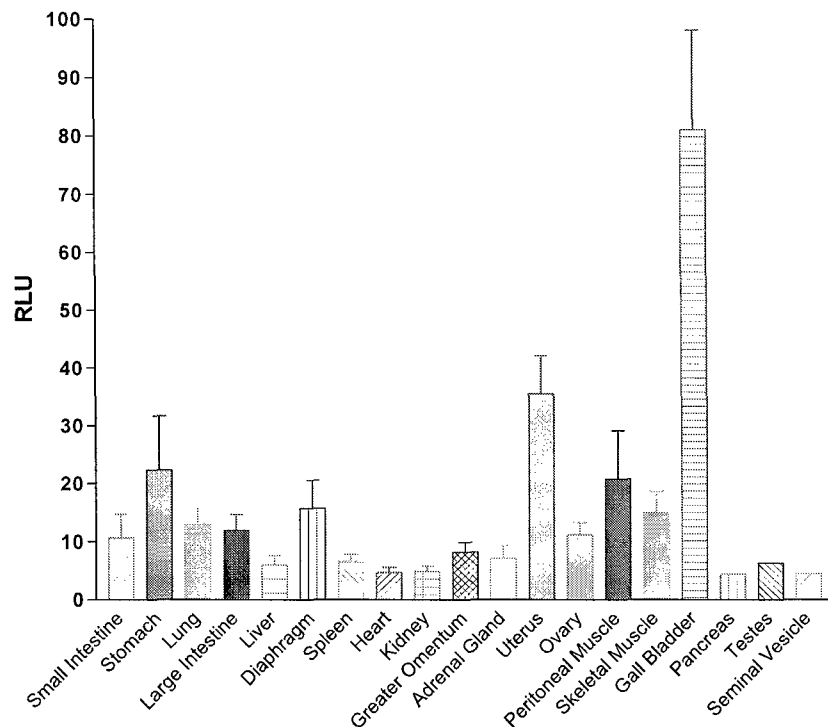
**Figure 3. HE4-766 and -652 are active in several epithelial ovarian cancer cell lines.** Epithelial ovarian cancer cell lines (A) and non-ovarian and normal cells (B) were transfected with either pHE4-766 or pHE4-652. CMV- $\beta$ -gal was cotransfected to control for transfection efficiency. Values (represented as a percentage of SV40 promoter driven luciferase activity), and SV40 were normalized to 100%. Results represent the mean of 2 independent experiments. Error bars represent SEM. (A) pHE4-766 is more active than pHE4-652 in all ovarian cancer cell lines. (B) Both pHE4-766 and pHE4-652 have minimal activity (<5%) in non-ovarian and normal cell lines. NHF; Normal human fibroblast, IOSE; Immortalized ovarian surface epithelium, MCF7; Breast cancer, NPA; Thyroid cancer, HeLa; Cervical cancer.

**Task 3 *In vivo* analysis of optimal promoter in a mouse model system.**



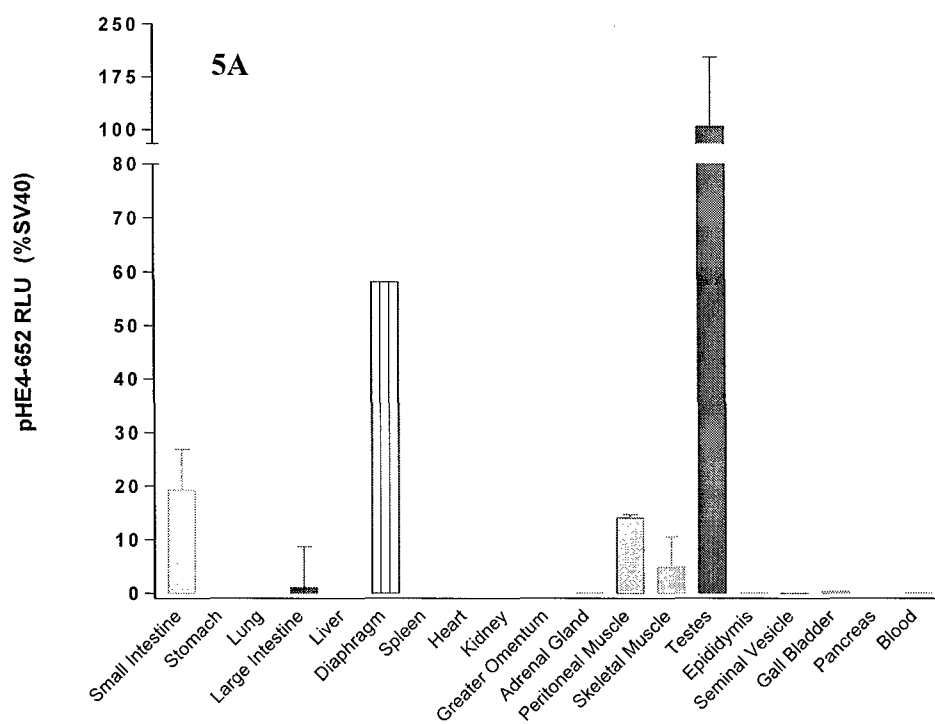
In order to evaluate the efficacy of a candidate promoter, it is important to determine the relative activity of the promoter in normal cells *in vivo*. Therefore, we performed a peritoneal transfection of a constitutively active SV40 promoter-driven luciferase reporter gene construct pGL3-control (Promega) complexed with the transfection lipid FuGENE6 (Roche, Indianapolis, IN). Lipid:DNA complexes were injected into the peritoneum of each mouse and allowed to incubate for 24 hours.

Following approved animal protocols and procedures, mice were then sacrificed, blood and peritoneal organs harvested, and tissues frozen on dry ice. Tissues were homogenized in reporter lysis buffer, centrifuged, and supernatants isolated for luciferase and protein assays. Luciferase activity was determined using a T20/20 luminometer (Turner Designs, Sunnyvale, CA) and values were expressed as relative luciferase units after normalizing to protein concentration and subtracting out minor background blood luciferase values. The maximum luciferase activity of each peritoneal organ (e.g., liver, spleen, kidney, etc) was determined. SV40-driven luciferase was observed in all peritoneal organs examined, though each tissue displayed differential usage of the SV40 promoter (**Figure 4**).



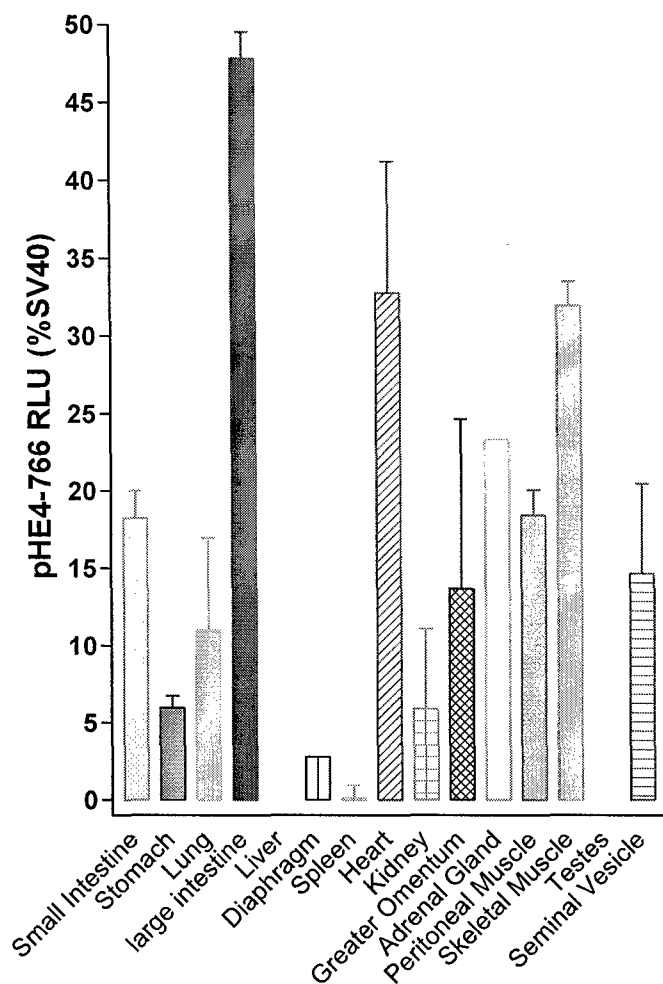
**Figure 4. *In vivo* transfection of SV40-Luciferase.** SV40-driven luciferase was seen in all peritoneal organs examined. Promoter activity for SV40 luciferase in each tissue type was expressed as relative luciferase units (RLU) after normalizing to protein concentration and subtracting out background luciferase values in the blood.

Having established maximum luciferase values for each tissue, we next determined the activity of pHE4-652 relative to that of the constitutively active SV40 promoter. Mice were injected with the pHE4-652-luciferase construct according to the procedure described above. Twenty-four hours later, peritoneal organs were harvested, analyzed, and compared to that of SV40-luciferase. No or very low pHE4-652 luciferase activity was seen in many tissues, with the exception of small intestine, muscles, and testes (Figure 5A). pHE4-766 was active in most tissues (Figure 5B).



**Figure 5A. *In vivo* transfection of pHE4-652 -Luciferase.** pHE4-652 was transfected with Fugene6 for 24hrs. Minimal activity was seen in many tissues except for muscle tissues, small intestine, and testes. pHE4-652 activity was also expressed as a percentage of SV40 luciferase activity after normalizing to protein concentration, subtracting out background blood luciferase values, and dividing by SV40 luciferase for each tissue.

Analysis of the promoter sequences revealed several transcription factor binding sites that recruit muscle-targeting transcription factors (MyoD, MTBF) as well as intestinal factors (GKLF). We hypothesize that these sites may be responsible for the activity of these HE4 promoters in muscle and intestinal tissues, and we will test this possibility by mutating these residues and examining the effect of the mutations on promoter activity in these tissues. Minor mutations within the HE4 promoter will hopefully provide optimal promoter activity and tightly ovarian cancer specificity. Of special interest is the activity of pHE4-652 in the testes. The HE4 gene was originally described in the male reproductive tract (2) and so it is not surprising that the promoter retains some activity in the testes. However, this activity should not compromise our goal of using the HE4 promoter for ovarian cancer gene therapy.



**Figure 5B. pHE4-766 activity *in vivo*.** *In vivo* transfection of pHE4-766. pHE4-766 was transfected with Fugene6 for 24hr. pHE4-766 activity was also expressed as a percentage of SV40 luciferase activity after normalizing to protein concentration, subtracting out background blood luciferase values, and dividing by SV40 luciferase for each tissue. Activity was seen in all tissues tested (liver and testes not examined).

**FUTURE DIRECTIONS:**

By providing that the novel promoters identified in Project 4 are expressed in normal ovarian epithelium, it seems likely the constructs could be used to target oncogenes specifically to the mouse ovarian epithelium and thus be useful in the future for developing a transgenic model for epithelial ovarian cancer. In addition, we will examine adenovirus or folic acid delivery of pHE4-766 and pHE4-652 *in vivo*. We have cloned the HE4 promoter upstream of the therapeutic gene HSV-tk and have begun creating ovarian cancer cell lines that stably express HSV-tk under the control of the HE4 promoter. These ovarian cancer cell lines will be used in ovarian cancer xenograft experiments. Those experiments will be conducted throughout this year and next. Overall, we believe the results of this study will support the further development of the HE4 promoter for clinical use as a gene therapy for ovarian cancer.

**REFERENCES:**

1. Berry NB, Cho YM, Harrington MA, Williams SD, Foley J, Nephew KP 2004 Transcriptional targeting in ovarian cancer cells using the human epididymis protein 4 promoter. *Gynecologic Oncology* 92:896-904
2. Kirchhoff C, Habben I, Ivell R, Krull N 1991 A major human epididymis-specific cDNA encodes a protein with sequence homology to extracellular proteinase inhibitors. *Biol Reprod* 45:350-7

**Core A: Animal Models**  
**(PI: Robert M. Bigsby, Ph.D)**

**INTRODUCTION:** Briefly, one paragraph, describe the subject, purpose and scope of the research.

The goal of the Animal Models Core was to test the effectiveness of mutated genes developed in Projects 1-3 for their ability to enhance therapeutic index in an in vivo setting. Cells harboring the mutant genes were to be grown as tumors in athymic mice and the mice would be treated with a chemotherapeutic agent or with X-irradiation. Dose-response studies were planned to determine the sensitivity of the altered tumors and this would be compared to the sensitivity of tumors from parent cell lines. Due to several technical difficulties, the engineered cell lines were not produced in Projects 1-3 and therefore different strategies were developed and the emphasis of the Core was shifted towards development of imaging techniques useful in following xenograft animal models in preclinical studies. The techniques of micro-PET/micro-CT were tested for their ability to follow growth and treatment responses of xenograft tumors. Another imaging system was developed to follow growth of tumors formed from cells harboring a luciferase reporter gene. The luciferase system is anticipated to be useful for assessing the tissue specificity of gene promoters being developed in Project 4. In addition, the xenograft models for which baseline data had already been worked out were used to test new strategies of tumor growth suppression using the target genes under study in Project 1. In the extension year, we have developed a new mouse model of ovarian cancer based on conditional deletion of suppressor genes and conditional expression of an oncogene. These mice will serve as genetically relevant preclinical models for testing novel therapeutics and investigating ovarian tumor biology.

**BODY** describe the research accomplishments associated with each task outlined in the approved Statement Of Work

*Task 1: Determine baseline chemo- and radio-sensitivity of control tumor lines.*

We established a tumor cell line from the culture cell line SKOV3. The cultured cells were injected subcutaneously into athymic mice. After 12 weeks, tumors were established in 2 of 4 animals. These tumors were serially passaged in athymic mouse hosts and then dispersed for cell culture. The cultured cell lines coming from these tumors were characterized for cytokeratin and vimentin expression. After expansion of the cell lines they were tested for their ability to form tumors in mice. One cell line formed tumors within 4-5 weeks after injection of 1 million cells sc; this line has been designated SKOV3x.

Hey C2 cells were already tumorigenic in athymic mice.

We developed the necessary baseline data on therapeutic responses of parent cell lines, SKOV3x and Hey C2. This baseline data are necessary for comparison in experiments using the engineered sublines to be submitted to the Animal Core from the projects.

Experiments with cisplatin indicated that the maximal response was achieved with 4 mg/kg administered at 4 day intervals for 4 doses to mice carrying either SKOV3x or Hey C2 tumors. Tumor growth was inhibited approximately 60% and 80% for SKOV3x and Hey C2 tumors, respectively.

X-irradiation experiments using grafted SKOV3x cells were performed. A dose of 9 Gy produced a dramatic decrease in growth of the tumors, increasing tumor volume doubling time from 6 to 14 days in one experiment and from 6 to 21 days in another; a dose of 6 Gy produced an intermediate growth rate. These preliminary experiments allowed us to develop the system for X-irradiation and to define the dose range for experimental tests in engineered cells.

*Task 2: Establish parameters for in vivo induction of gene expression.*

Although it was originally proposed to use an ecdysone-inducible promoter to drive expression of the mutant target genes, it was found that another system, the tetracycline controlled promoter worked well in culture. Since the tet-on system would be much more economical, it was decided that this would be the used throughout. Three attempts to grow cells expressing an inducible form of mutated APE have failed. Cells stably transfected with empty vector did grow. Apparently, control of expression of the mutant APE did not allow for null expression in the absence of an exogenous stimulator. Because we were unable to get a clean negative control, the idea of using inducible expression vectors has been scrapped

*Task 3: Establish tumor lines from engineered cell sublines supplied from Projects 1-3.*

Development of a cell line expressing the mutant p27 (Project 3) has been problematic. The mutant gene apparently arrests the cells, even during a transient transfection. An experiment was performed to determine the fate of the transiently transfected cells *in vivo*. Transfection of 293 cells with empty vector, reduced their ability to form tumors slightly compared to non-transfected cells. Transfection with the mutant p27 produced cells that did not make tumors. An inducible gene expression system is required for further study of this mutant but attempts to produce such a system have been fruitless.

No sublines were supplied from the projects; other approaches were pursued to test the principles underlying the hypothesis in Project 1 (see below).

*Task 4: Perform chemotherapy and irradiation dose response studies on engineered cell lines.*

We established basal growth rates and responses to X-irradiation in tumors derived from xenograft of parent cell lines using both subcutaneous and intraperitoneal inoculations. Although there were no engineered cell lines derived in any of the projects, we applied two approaches to this task: 1. The efficacy of the PET/CT imaging system was tested with tumor xenografts. 2. The growth inhibitory effects of gene knock-down was tested using siRNA to one of the target genes of Project 1. In addition, we continued studies on the usefulness of the luciferase imaging system for following growth of intraperitoneal tumors.

*PET/CT imaging:* Although the mathematical means of assessing the PET images is still under study, the first pass analysis was promising. We observed that  $^{18}\text{F}$ FDG glucose utilization was found to be significantly reduced in 9 Gy irradiated tumors versus controls 20 days post-irradiation. This is the first step towards our long term goal of using PET to non-invasively follow ovarian tumor response post-treatment after X-rays and/or chemotherapy.

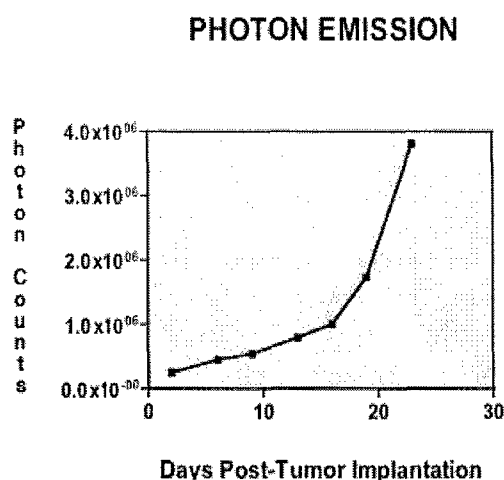
Caliper measurement of tumor volume was also compared to  $^{18}\text{F}$ -UDG uptake, with or without treatment (irradiation or cisplatin chemotherapy). There was a high degree of correlation between caliper measurement and PET volume for control tumors and tumors treated with either X-irradiation or cisplatin (r-squared estimated to be greater than 0.90). The tumor volume as measured by PET was significantly less than that of control tumor at 20 days post-irradiation (9 Gy); however, tumor volumes as determined by caliper measurement were significantly different at 10 days post-irradiation. Mathematical treatment of the PET data is still under development and it is hoped that the sensitivity of the system can be increased with better analysis.

*Effects of siRNA to APE (Project 1, target gene):* Experiments performed in vitro in Project 1 showed that knock-down of the DNA repair enzyme, APE using a single transfection of siRNA oligonucleotide would inhibit cell growth. The growth of cells treated with APE siRNA was tested in xenograft. In the first experiment, it was found that there was a dramatic block of tumor growth as long as APE protein was diminished, i.e. during the 15 day observation period. In subsequent

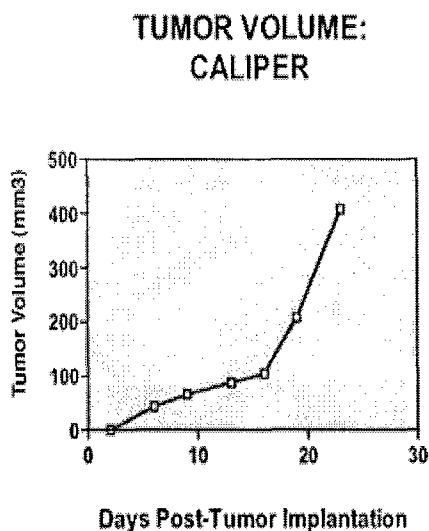


experiments, the growth inhibition was not nearly as dramatic, lasting only 1 week, but resumption of tumor growth correlated to renewed expression of APE. These results indicate that APE is a valid target for control of ovarian tumor growth through gene manipulation.

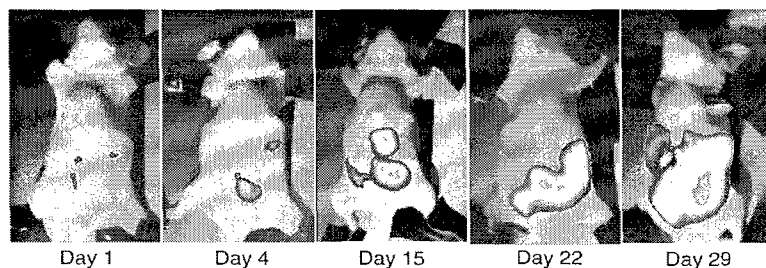
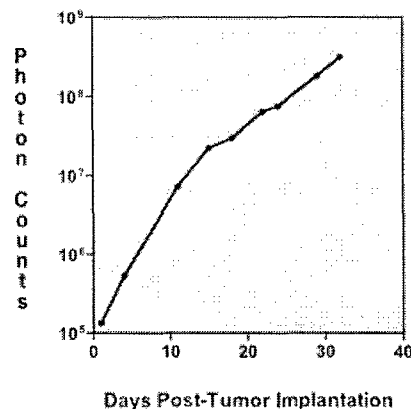
*Luciferase Imaging:* Cell lines were developed from HeyC2 cells in which the firefly luciferase gene was stably transfected. These cell lines were used in xenograft to determine their usefulness for following tumor growth in the abdominal cavity. Tumor cells were injected subcutaneously. By simultaneously imaging tumors for luciferase expression and measuring their volumes with calipers (Fig. 1a & b), mathematical methods were derived for analysis of luminescence intensity and this was correlated with time of tumor growth. Imaging tumors growing intraperitoneally (Fig. 2), a linear progression of tumor volume increase was detected over the course of 32 days following cell inoculation. At the time of necropsy, tumor location and relative mass correlated well with the images. This model will be extremely useful in following orthotopic growth of ovarian tumor xenografts.



**Fig. 1a. Photon emission by the luciferase-expressing tumor increases over time as the tumor grows.** The total signal intensity at the tumor site was quantitated using the NightOWL imaging system. The photon counts are plotted versus days after tumor cells were injected s.c. The profiles of the photon emission graph and tumor volume graph are similar.



**Fig. 1b. Growth of the luciferase-expressing s.c. tumor as determined by caliper measurements.** The volume of the tumor was calculated from caliper measurements and plotted versus time in days. Increased tumor volume is related to increased photon emission.

**Luciferase Expression in Intraperitoneal Hey C2 Tumors****PHOTON EMISSION FROM I.P. TUMOR**

**Fig. 2.** The development of intraperitoneal tumors can be followed even at very early time points using *in vivo* bioluminescence imaging. A nude mouse was injected i.p. with  $3 \times 10^5$  HEYC2luc cells. The next day the mouse was imaged using the NightOWL system as described earlier. Further images were obtained at various time points until the animal displayed significant abdominal distension.

**Task 5: Statistical analysis of results**

As indicated above, mathematical and statistical analysis of the imaging systems were performed. Analysis of the APE siRNA experiments showed that tumor growth was significantly decreased in the siRNA-treated groups.

**New Tasks for Year 4 (extension)**

**Task 6:** Analyze the ability of Ovarian-specific Promoters (Project 4) to drive tissue-specific expression of a reporter gene.

The goal of Project 4 was to identify and test the effectiveness of gene promoters that would drive transcription in ovarian surface epithelium or tumor cells, either exclusively or to a much greater degree than in other tissues. One such gene promoter is under study, HE4 and promoter-reporter constructs have been made. Transgenic animals will be used to follow the expression of the luciferase reporter under the HE4 promoter. The luciferase imaging system described above will be used to detect this expression. Production of these transgenic animals has been supported by another granting mechanism but they are not yet available.

**Task 7: Develop new models of ovarian cancer for preclinical studies.**

Although the xenograft models described in our research are useful for studying the effects of treatment in late stage cancers, they are not at all useful for study of early stages of the disease. In the extension year we set out to develop a Cre/Lox model of ovarian cancer based on the work of Flesken-Nikitin et al, 2003 in which the expression of the two tumor suppressor genes, Rb-1 and p53, was conditionally knocked out in ovarian surface epithelial cells following intrabursal inoculation of a viral vector for Cre recombinase expression. All animals treated in this fashion developed ovarian tumors within 8 months.

One of the problems with the Flesken-Nikitin model is that it depends on the conditional deletion of both p53 and Rb-1, but loss of Rb-1 is only a rare occurrence in human ovarian cancer. Thus, genetically, this model does not reflect the human situation. On the other hand, the intermediate signalling protein, K-Ras is often mutated to its oncogenic form K-RasG12D in ovarian

cancer. We will modify the mouse model using a transgenic animal that carries a copy of K-RasG12D that is expressed conditionally upon Cre-mediated excision of a floxed STOP signal (floxed-STOP-K-RasG12D). The floxed-p53 and floxed-STOP-K-RasG12D mice will be interbred to produce animals in which oncogenic K-RasG12D is expressed conditionally and p53 is also deleted conditionally upon application of viral vector for Cre recombinase. It is hypothesized that the combination of these two gene alterations will result in neoplastic transformation of the ovarian surface epithelial cells and that tumors will grow more rapidly due to the presence of oncogenic K-Ras.

We have acquired all of the necessary strains of mice for these studies: floxed-Rb; floxed-p53; floxed-Rb/floxed-p53; and floxed-STOP-K-RasG12D. We developed colonies of each of these strains and have cross-bred the floxed-p53 and the floxed-STOP-K-RasG12D mice to produce animals that are hemizygous floxed-STOP-K-RasG12D and homozygous floxed-p53. The double transgenic mice, floxed-Rb/floxed-p53, will serve as the positive controls for the experimental induction of tumors.

We also acquired animals that are transgenic for floxed-STOP-lacZ, a reporter gene that is expressed only after removal of a STOP sequence by Cre-recombinase. These animals have served as indicators for the efficacy of the viral vector, Ad5CMVCre. At 48 h after intrabursal application of the vector, cells on the surface of the ovary expressed lacZ, as judged by development of chromogen on the surface of ovaries following incubation in X-gal substrate.

To date we have treated 32 double transgenic, floxed-p53/floxed-Rb mice with Ad5CMVCre; one of those mice had an ovarian tumor of 1.5 cm diameter after 16 weeks. Within the last month of the grant, we have produced and treated 10 double transgenics, floxed-p53/floxed-STOP-K-RasG12D mice and we are following these animals for tumor production.

We have sought and received a small grant to continue these studies with the conditional knockout mice. We anticipate further modification of the model by cross-breeding in a Cre-dependent luciferase gene that will allow us to non-invasively monitor tumorigenesis. These animal models will be amenable to both preclinical studies of novel therapeutics and studies of ovarian tumor biology.

#### REFERENCES CITED

Flesken-Nikitin A, Choi KC, Eng JP, Shmidt EN, and Nikitin AY (2003) Induction of carcinogenesis by concurrent inactivation of p53 and Rb1 in the mouse ovarian surface epithelium. *Cancer Res* 63(13): 3459-3463

**KEY RESEARCH ACCOMPLISHMENTS****Project 2**

- Validation of the efficacy of a peptide-based inhibitor to DNA-PK in lowering ovarian cancer cell growth following ionizing radiation
- Establishment of adenoviral expression of Ku80
- As an extended study, we found out that EGF receptor contributes to drug resistance of human germline tumors

**Project 3**

- Wild-type and mutant p57<sup>Kip1</sup> proteins both interact with CDK2; however the amount of CDK2 associated with the mutant p57<sup>Kip2</sup> is reduced.
- p27<sup>Kip1/T187A</sup>, the stabilized version of the p27<sup>Kip1</sup> protein, is a more effective inhibitor of tumor cell growth when compared to wild-type p27<sup>Kip1</sup>, wild-type p57<sup>Kip2</sup> or mutant p57<sup>Kip2</sup>.
- Folate-PEG-poly-L-lysine complexes can be successfully used to transfect plasmid DNA expression vectors to ovarian cancer cells *in vitro*.
- *In vivo* bioluminescent imaging of i.p. tumors is a possible new tool to study ovarian cancer tumor growth/regression in a murine model system.

**Project 4**

- Published HE4 promoter findings (*Berry et al. Gynecologic Oncology* 2004 Mar;92(3):896-904).
- Selected pHE4-766 as an additional promoter candidate
- Identified transcription factors that confer HE4 activity and specificity
- Established *in vivo* system to analyze HE4 activity in normal tissues
- Cloned 13 HE4 promoter deletion constructs
- Compared 13 constructs in ovarian, non-ovarian, and normal cells
- Compared transcription factors between original HE4-652 and 766
- *In vivo* analysis of 652 and 766
- Patent application (*in progress*)

**Core A**

- Established protocols for transferring ovarian cell lines into athymic mouse hosts
- Determined baseline responses of SKOV3x cell tumors to X-irradiation
- Determined baseline responses of SKOV3x cell tumors and Hey C2 cell tumors to cisplatin
- Determined the effect of transient transfection of mutant p27 on tumor take
- Established a non-invasive method for following intraperitoneal tumor growth using microPET and determined efficacy of this imaging technique for following therapeutic response
- Established the effectiveness of the siRNA technology to reduce target gene expression and thereby affect cellular growth
- Established APE as a valid target for gene therapy of ovarian cancer
- Established protocols for *in vivo* imaging of intraperitoneal tumors expressing luciferase
- Established colonies of transgenic animals in which tumor suppressor genes, p53 and/or Rb, are conditionally deleted upon infection with viral vector expressing Cre recombinase.

- Derived double transgenic mice in which the tumor suppressor gene, p53 is deleted and the oncogene, K-RasG12D is expressed upon infection with viral vector expressing Cre recombinase.

## **REPORTABLE OUTCOMES**

### **Project 2**

Park, S-J, Armstrong, S.A., Kim, C-H., Robertson, K., Kelley, M., and Lee, S-H. (2005) Lack of EGF receptor contributes to the drug sensitivity in human germline cells. **British J. Cancer** 92(2): 334-341.

Young-Ju Lee, Su-Jung Park, Samantha L.M. Ciccone, Chong-Rak Kim, and Suk-Hee Lee (2005) An *in vivo* analysis of MMC-induced DNA damage and its repair. **Carcinogenesis** (accepted)

### **Project 4**

- Published HE4 promoter findings (Berry *et al. Gynecologic Oncology* 2004 Mar;92(3):896-904).
- Patent application
- SGI Award
- Brian McCarthy 2004 Midwest Regional Molecular Endocrinology poster

## **CONCLUSIONS**

### **Project 2**

The overall goal of this proposal is to explore the role of DNA-PK in the development and progression of ovarian cancer. Since DNA-PK is a DNA repair factor as well as involved in damage signaling pathway, levels of DNA-PK activity among ovarian cancer would contribute to their drug resistance and also provide the basis for selection of patients for treatment with chemotherapy drugs.

From the three-year funding, we concluded that a peptide-based inhibitor preventing DNA-PKs from forming a complex with Ku70/Ku80 significantly lowered DNA-PK activity. Furthermore, treatment of these ovarian cancer cells with target peptide significantly lowered the cell growth only in the presence of ionizing radiation, indicating that the peptide-based inhibitor exhibited a positive effect of on lowering drug resistance by specifically targeting DNA-PK *in vivo*. This result also validates a physiologic role for DNA-PK in chemotherapy drug resistance of ovarian cancers.

### **Project 3**

Using *in vivo* bioluminescence imaging we have shown that the number of photons emitted by a luciferase-expressing tumor is related to the volume of the tumor. We also demonstrate that this is a very sensitive optical imaging method which is able to detect small numbers of tumor cells within an animal. The technique allows one to monitor the *in vivo* dynamics of tumor growth both efficiently and economically. This i.p. model of ovarian cancer will be a valuable tool to evaluate the effectiveness of novel therapeutic agents.

**Project 4**

This information has allowed us to determine that pHE4-766 candidate promoter has the potential to give higher levels of reporter gene activity in ovarian cancer cells compared to that of pHE4-652. However, *in vivo* analysis of the pHE4-766 promoter revealed non-specific activity contrary to *in vitro* data. pHE4-652, on the other hand, remains promising, as fewer peritoneal organs displayed promoter activity. Minor adjustment/mutation of the non-specific transcription factors in pHE4-652 may create the optimal promoter and experiments to do so are currently underway.

Some of the findings outlined in this report will be included as part of future grant applications to extramural funding agencies, such as the National Institutes of Health. Furthermore, as described below, a novel ovarian epithelial promoter can be used in the future development of a transgenic animal model for ovarian cancer. This promoter will be a key component in generating our ovarian specific transgenic cassette. It appears that the additional base pairs upstream of pHE4-652 are necessary to drive the highest ovarian cancer activity, but also appear to be non-specific enhancers.

For the "so what section", which evaluates the knowledge as a scientific or medical product to also be included in the conclusion of this report, we would like to point out that currently there are few animal models for epithelial ovarian cancer, which is a severe limitation both for understanding the basic biology of this devastating disease and testing novel therapeutics for epithelial ovarian cancer. It is clear that the lack of promoters to drive oncogene expression specifically in the ovarian epithelium is a key limitation, if not the major barrier, to developing a transgenic mouse model of the disease.

**Core A**

Expression of long-lived mutant form of p27 blocks tumor growth.

Intraperitoneal tumors can be followed by either micro-PET/micro-CT

Tumor growth can be inhibited by reducing APE expression.

Luciferase expression by intraperitoneal tumor cells can be monitored non-invasively and correlated to tumor growth.

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Young-Ju Lee, Su-Jung Park, Samantha L.M. Ciccone, Chong-Rak Kim, and **Suk-Hee Lee** (2005) An *in vivo* analysis of MMC-induced DNA damage and its repair. *Carcinogenesis* (accepted)

## ABSTRACTS

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## **PERSONNEL**

### **Project 1**

Mark Kelley, Ph.D.  
Meihua Luo  
Yi Xu  
Lucy Zhu

### **Project 2**

Suk-Hee Lee, Ph.D.  
Anita Riemen  
Jong Won Kim  
Suzanne Inchauste

### **Project 3**

Maureen Harrington, Ph.D.  
Mark Goebel, Ph.D.  
Bernadette Allison  
Suzanne Inchauste  
Lee Ott

### **Project 4**

Kenneth P. Nephew, Ph.D.  
Phillip Abbosh  
Davud Bower  
Yamei Cheng  
Min Choi  
Teresa Craft  
Mei Fan, Ph.D.  
John Foley, Ph.D.  
Phyllis Gibbs  
Sheryl Wilson

### **Core A**

Robert M. Bigsby, Ph.D.  
Chang-Yi Xie  
Marc Mendonca, Ph.D.  
Kathleen Comerford  
Bernadette Allison  
Helen Jan Chin-Sinex

### **Core B**

Stephen D. Williams, M.D.  
Constantin Yiannoutsos, Ph.D.  
Menggang Yu, Ph.D.

## APPENDICES

### Manuscripts since last submission

Park, S-J, Armstrong, S.A., Kim, C-H., Robertson, K., **Kelley, M.**, and **Lee, S-H.** (2005) Lack of EGF receptor contributes to the drug sensitivity in human germline cells. ***British J. Cancer*** 92(2): 334-341.

Tell, G., Damante, G., Caldwell, D. and **Kelley, M.R.** (2005) The intracellular localization of APE1/Ref-1: More than a passive phenomenon? *Antioxidants and Redox Signaling* 7: 365-382.

Young-Ju Lee, Su-Jung Park, Samantha L.M. Ciccone, Chong-Rak Kim, and **Suk-Hee Lee** (2005) An *in vivo* analysis of MMC-induced DNA damage and its repair. ***Carcinogenesis*** (accepted)

# Lack of EGF receptor contributes to drug sensitivity of human germline cells

S-J Park<sup>1,2</sup>, S Armstrong<sup>1</sup>, C-H Kim<sup>1</sup>, M Yu<sup>3</sup>, K Robertson<sup>4</sup>, MR Kelley<sup>1,4</sup> and S-H Lee<sup>\*,1,2</sup>

<sup>1</sup>Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA; <sup>2</sup>Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN 46202, USA; <sup>3</sup>Department of Medicine-Biostatistics, Indiana University School of Medicine, Indianapolis, IN 46202, USA; <sup>4</sup>Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN 46202, USA

Germline mutations have been associated with generation of various types of tumour. In this study, we investigated genetic alteration of germline tumours that affect the drug sensitivity of cells. Although all germline tumour cells we tested were hypersensitive to DNA-damaging drugs, no significant alteration was observed in their DNA repair activity or the expression of DNA repair proteins. In contrast, germline tumours expressed very low level of epidermal growth factor receptor (EGFR) compared to drug-resistant ovarian cancer cells. An immunohistochemical analysis indicated that most of the primary germline tumours we tested expressed very low level of EGFR. In accordance with this, overexpression of EGFR in germline tumour cells showed an increase in drug resistance, suggesting that a lack of EGFR, at least in part, contributes to the drug sensitivity of germline tumours.

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Germline mutations are associated with generation of various tumours. Previous studies indicated that germline cells were hypersensitive to DNA-damaging agents (Cagnoli *et al*, 1998). Although the mechanisms, of drug resistance are still poorly understood, an increased rate of DNA adduct removal appears to be associated with drug resistance in various human cancers (Lai *et al*, 1988; Dinapoli *et al*, 1993; Johnson *et al*, 1994; Eastman and Schulte, 1998). Drug-resistant human tumours have been shown to express higher levels of nucleotide excision repair (NER) proteins such as XPA, XPB (Lai *et al*, 1988; Eastman and Schulte, 1998), ERCC1, and cockayne syndrome group B (CSB) (Lai *et al*, 1988). Also, altered expression of genes involved in O-6-alkyltransferase-mediated direct DNA repair (O-6-methylguanine DNA methyl transferase, MGMT) or base excision repair pathway also contributes to drug resistance of cancer cells (Chetsanga and Lindahl, 1979; Doetsch and Cunningham, 1990; Cohen *et al*, 1991; Demple and Harrison, 1994; Gill *et al*, 1996; Deutsch *et al*, 1997; Asagoshi *et al*, 2000; Bielas and Heddle, 2000; Dobson *et al*, 2000; Evans *et al*, 2000). Defects in mismatch repair (MMR) are associated with cisplatin resistance by contributing to increased replication bypass of cisplatin adducts and to a drug-tolerant phenotype (Hansen *et al*, 1998; Karahalil *et al*, 1998; Hansen and Kelley, 2000; Limp-Foster and Kelley, 2000; O'Neill, 2000). Therefore, loss of MMR proteins such as hMLH1 leads to resistance of tumour cells to a variety of DNA-damaging agents, including bifunctional alkylating and monofunctional methylating agents

(Rosenquist *et al*, 1997; Waters *et al*, 1999; Zharkov *et al*, 2000) such as cisplatin and N'-methyl-N-nitrosourea (Koi *et al*, 1994; Fishel and Kolodner, 1995; Fink *et al*, 1996, 1998; Drummond *et al*, 1996).

In addition to genetic alteration of DNA repair genes, altered drug transport, increased metallothionein or glutathione levels, mitochondrial alterations, and altered DNA adduct formation have been reported to contribute to drug resistance of human cancers (Graves *et al*, 1992; Mello *et al*, 1996; Kuga *et al*, 1997; Leighton *et al*, 1997; Mitra *et al*, 1997; Umar *et al*, 1997). Growth factor receptor such as epidermal growth factor receptor (EGFR) is also amplified in many solid tumours (Arteaga, 2003). Introduction of a protein tyrosine kinase inhibitor selectively blocks proliferation of EGFR-expressing tumour cells, suggesting a role for EGFR in tumour cell growth (Lydon *et al*, 1998). Epidermal growth factor receptor is necessary for cisplatin-mediated apoptosis in tumour cells, suggesting a possible involvement of EGFR pathway in mediating the repair of drug-induced DNA damage(s) (Dixit *et al*, 1997). On the other hand, overexpression of EGFR family members suppressed the antiproliferative/cytotoxic activity of tumour necrosis factor (TNF)-alpha, suggesting that it may have an antagonistic role in TNF pathway (Perez and Donato, 1996; Hoffmann *et al*, 1998). Nonetheless, overexpression of EGFR observed in human breast/ovarian tumours is associated with poor prognosis with cancer patients (Baekelandt *et al*, 1999; Witters *et al*, 1999).

In this study, we investigated the genetic alteration of germline tumours such as altered DNA repair activity and/or damage signalling pathways that affect the drug sensitivity of cells. We found no significant change in NER activity or expression of DNA repair proteins in drug-sensitive germline cells compared to the drug-resistant ovarian cancer cells. Instead, the expression of a membrane receptor tyrosine kinase, EGFR, correlated with the

\*Correspondence: Dr S-H Lee, Indiana University Cancer Research Institute, 1044 W Walnut Street, Room 153, Indianapolis, IN 46202, USA; E-mail: slee@iupui.edu

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cells drug resistance. Drug-resistant cancer cells exhibited elevated level of EGFR expression, while drug-sensitive germline cells showed a lower EGFR expression. Overexpression of the EGFR gene significantly enhanced the cells drug resistance, suggesting that EGFR may be one of the contributing factors that affect drug resistance of cancer cells.

## MATERIALS AND METHODS

**Cell lines, cell culture, and drug treatment:** NT2/D1 cells were obtained from American Type Culture Collection (Rockville, MD, USA) and 833K and 64CP9 GCT cell lines were obtained from G Sledge (Indiana University School of Medicine, Indianapolis, IN, USA). PA-1 cells were derived from a human teratocarcinoma, human ovarian cancer cells (Hey) were from a peritoneal deposit of a cytoadenocarcinoma of the ovary (from G Mills, MD Anderson Cancer Center, Houston, TX, USA), and a normal ovarian epithelial cell (IOSE80) was obtained from JA Hurteau (Department of Obstetrics and Gynecology, University of Illinois at Chicago, Chicago, IL, USA). All germline and ovarian cells were maintained in MEM supplemented with 10% fetal bovine serum at 37°C in a CO<sub>2</sub> incubator, while IOSE 80 was maintained in MEM and 199/MCDB 105 (1:1) supplemented with 10% fetal bovine serum and EGF (10 ng ml<sup>-1</sup>).

**Germ cell tumours (GCTs):** Tissue sections of biopsy materials with disseminated GCTs were obtained from the Indiana University Medical Center, University Hospital, under an Indiana University Institutional Review Board approved protocol (IU Study No. 9908-47) as 4% buffered formaldehyde-fixed tissues embedded in paraffin blocks, which were sectioned at 3 mm and fixed onto slides. Diagnosis was made from morphological examination of H&E-stained sections of biopsy material.

**Proteins, plasmids, chemicals, and antibodies:** Glutathione-S-transferase (GST) fusion form of c-Jun protein containing residues 1–79 of human c-Jun was overexpressed from *Escherichia coli* and purified using glutathione-agarose affinity column chromatography as described previously (Park et al, 2001). [ $\gamma$ -<sup>32</sup>P]ATP (4500 Ci mmol<sup>-1</sup>) was obtained from ICN. Adriamycin, EGF mitomycin C (MMC), and cisplatin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Antibodies to EGFR, proliferating cell nuclear antigen (PCNA), c-Abl, Ku70/80, the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), JNK1 and/or JNK1/2 were obtained from either Santa Cruz Biotechnology (Santa Cruz, CA, USA) or Pharmingen (San Diego, CA, USA).

**Cell survival assay:** To examine drug resistance of cells, cells ( $1.0 \times 10^4$  cells well<sup>-1</sup>) were plated in a 96-well plate and incubated for 24 h. Cells were treated with drugs and further incubated at 37°C and 5% CO<sub>2</sub> for 72 h. After 72 h incubation, cell survival was measured using a colorimetric cell survival assay from Boehringer Mannheim (MTT Cell Proliferation Kit). Alternatively, clonogenic assay was used to measure the ability of cells to form colonies on 100 mm<sup>2</sup> tissue culture dishes following treatment with ionising radiation or cisplatin. Controls consisted of cells untreated with peptides or DNA-damaging agent, or with neither. Cells were continuously exposed for 5 days to the indicated concentrations of the peptide, and colonies were stained with crystal violet and then colonies greater than 50 cells were counted. Each point represents mean values  $\pm$  s.e., each conducted with triplicate plates.

**Immunohistochemistry:** Tissue sections were visualised for EGFR expression using an anti-EGFR monoclonal antibody (Santa Cruz Biotech., Santa Cruz, CA, USA). The Dako Universal Staining system (Dako Corp., Carpinteria, CA, USA) was used to automate the immunostaining procedure (Robertson et al, 2001). Sections were treated with 3% H<sub>2</sub>O<sub>2</sub> for 10 min and incubated with an anti-EGFR antibody (1:1000) for 25 min, the biotinylated goat anti-mouse antibody IgG secondary antibody for 10 min, streptavidin-

horseradish peroxidase for 10 min, and diaminobenzidine for 5 min, according to Dako recommendation and empiric determination.

**JNK immunocomplex assay:** For JNK assay, cells were grown in culture media containing 0.5% fetal bovine serum for 16 h prior to the treatment with EGF or genotoxic agents. Cells were washed in ice-cold phosphate-buffered saline (PBS) and 0.5 ml of JNK lysis buffer (25 mM HEPES, pH 7.5, 0.3 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.5% Triton X-100, 20 mM  $\beta$ -glycerophosphate, 1 mM sodium vanadate, 0.1 M okadaic acid, 1 mM phenylmethylsulphonyl fluoride, 20  $\mu$ g ml<sup>-1</sup> aprotinin, 50  $\mu$ g ml<sup>-1</sup> leupeptin, and 10  $\mu$ M pepstatin) added to the dishes (150  $\times$  25 mm) before scraping. After 30 min incubation on ice, insoluble materials were removed by centrifugation for 30 min at 12 000 r.p.m. JNK activity was determined by an immunocomplex assay essentially as described (Litz-Jackson et al, 1992; Duyster et al, 1995). Briefly, cell extracts (200  $\mu$ g) were mixed with 1.5  $\mu$ l of anti-JNK1/JNK2 polyclonal antibody for 1 h, and then 15  $\mu$ l of protein A-Sepharose beads was added and further incubated for 3 h at 4°C. The immunocomplex was washed three times with JNK lysis buffer and once with JNK kinase reaction buffer (20 mM HEPES pH 7.5, 10 mM MgCl<sub>2</sub>, 7 mM MnCl<sub>2</sub>, 1 mM EGTA, 1 mM sodium fluoride, 1 mM sodium vanadate, and 1 mM DTT). The precipitate was then resuspended in 30  $\mu$ l of JNK reaction buffer containing 2  $\mu$ g of GST-c-Jun (Park et al, 2001) and 50  $\mu$ M ATP and the reaction was initiated by the addition of 1.0  $\mu$ l of [ $\gamma$ -<sup>32</sup>P]ATP (45 000 Ci mmol<sup>-1</sup>). After incubation for 20 min at 30°C, the reaction was terminated by the addition of 8  $\mu$ l of 4  $\times$  SDS sample buffer (Laemmli, 1970) and heating to 95°C for 5 min. Samples were analysed on a 12% SDS-PAGE.

**Western blot analysis:** Extracts (40  $\mu$ g) from various ovarian cancer cells were loaded onto a 6 or 10% SDS-PAGE, and following gel electrophoresis proteins were transferred to nitrocellulose membrane and immunoblotted with primary antibody followed by a peroxidase-coupled secondary antibody (Amersham) and an enhanced chemiluminescence (Amersham) reaction prior to visualisation on a Kodak-o-mat film.

**Transfection and selection of stable cell lines:** Cells were transfected with either pEGFR-GFP or pEGFP-N3 using Lipofect AMINE method (Life Technologies Inc.). Following antibiotic selection with G418 (600  $\mu$ g ml<sup>-1</sup>, Geneticin-Life Technologies, Gaithersburg, MD, USA), several EGFR-expressing clones were isolated and expanded into cell lines. Individual clonal lines expressing EGFR-GFP were established by plating a single cell into 96-well dishes. Cell clones expressing EGFR-GFP were utilised for the drug resistance study.

**Immunofluorescence microscopy:** PA-1 cells were grown on cover slides, washed twice with PBS, fixed in -10°C methanol for 5 min, air dried, and washed three times again with PBS. Fixed cells were incubated with an anti-EGFR polyclonal antibody (Santa Cruz Biotech.; 2  $\mu$ g ml<sup>-1</sup>) at room temperature for 1 h. After washing with PBS three times, cells were incubated for 1 h in the dark at room temperature with a 1:10 dilution of the secondary antibody (fluorescein-conjugated goat anti-mouse antibody; Oncogene Science) in PBS with 3% (w/v) milk. Following extensive washing with PBS (five times), slides were prepared using 90% glycerol in PBS and stored in the dark at 4°C. Images were collected using a CCD 4910 camera with NIH image on a Zeiss Axiophot microscope.

**In vitro NER activity:** Reaction mixtures (50  $\mu$ l) contained 0.2  $\mu$ g each of UV-irradiated (450 J m<sup>-2</sup>) pBS (3 kb) and nonirradiated pBS (4.5 kb), 40 mM creatine phosphate-di-Tris salt (pH 7.7), 1  $\mu$ g creatine kinase, 50 mM HEPES-KOH (pH 7.8), 70 mM KCl, 7.5 mM MgCl<sub>2</sub>, 0.5 mM DTT, 0.4 mM EDTA, 2 mM ATP, 20  $\mu$ M of dGTP, dCTP, dTTP, 8  $\mu$ M of [ $\alpha$ -<sup>32</sup>P] dATP (25 000 cpm pmol<sup>-1</sup>), 5  $\mu$ g of BSA, and increasing amount of cell extracts (150 and 300  $\mu$ g) from various cells (Stigger et al, 1998). After incubation for 3 h at 30°C, DNA was isolated from the reaction mixtures, linearized with

BamH1, and separated on a 1% agarose gel electrophoresis in the presence of  $0.5 \mu\text{g ml}^{-1}$  ethidium bromide. Repair products were analysed by both fluorography and exposure to X-ray film.

## RESULTS

### Germline cells exhibit hypersensitivity to DNA-damaging drugs

To analyse drug resistance of germline cells, four established germline tumour cells (PA-1, NT2/D1, 833K, and 64CP9) were compared with a normal ovarian epithelial cell line (IOSE80) and drug-resistant ovarian cancer cells (Hey) derived from a peritoneal deposit of a cytoadenocarcinoma of the ovary (Figure 1). The established ovarian cancer cells (Hey) showed a marked resistance to cisplatin treatment, while the germline tumour cells were remarkably sensitive to the drug treatment (Figure 1). All four germline tumour cells showed extreme sensitivity to cisplatin treatment ( $5 \mu\text{M}$ ) with a survival rate of less than 10%, whereas 80% of Hey cells survived under the same conditions. Meanwhile, a primary epithelial ovarian cell (IOSE80) showed a medium level of cell survival following cisplatin treatment (Figure 1). Adriamycin is a DNA-intercalating agent that causes DNA strand break damage, while MMC mainly causes DNA damage by forming a DNA crosslink. Similar to the cisplatin treatment, germline tumour cells were highly sensitive to both MMC and adriamycin treatment (Figure 1B and C).

### Drug sensitivity of germline cells correlates with the lack of EGFR expression

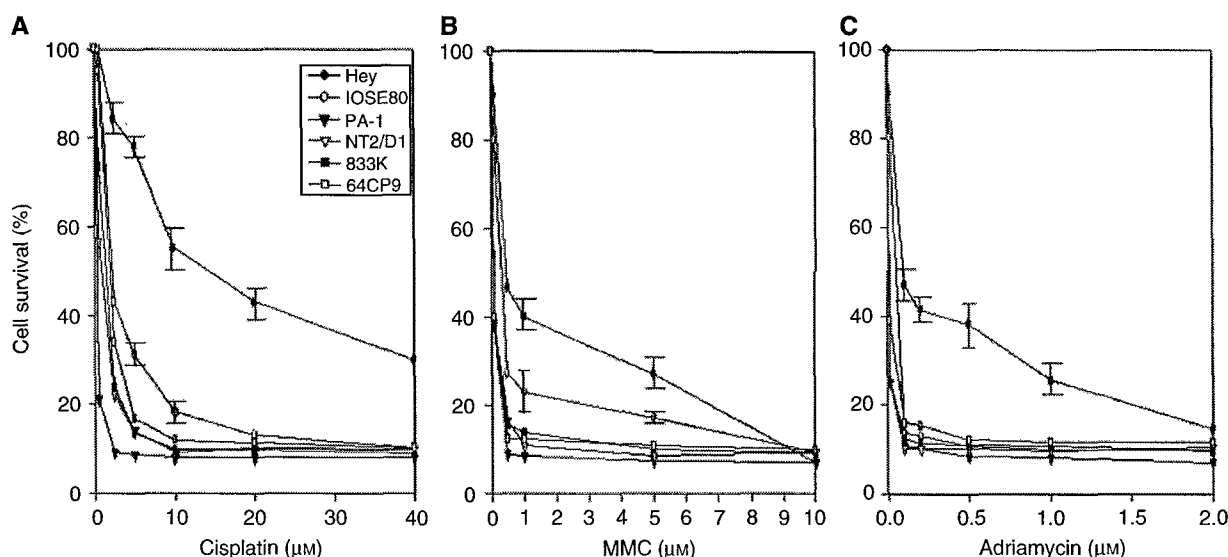
To better understand the hypersensitivity of germline tumour cells to DNA-damaging drug, we analysed expression of various proteins that are involved in the drug sensitivity of cells. No noticeable difference was observed between drug-sensitive germline tumour cells and a drug-resistant cell (Hey) in the expression of DNA repair proteins (PCNA, TFIIF, DNA-PKcs, and Ku70/80) (Figure 2). We noticed however some difference in the expression of DNA-PKcs (Figure 2), although this subtle difference was not consistently observed in multiple experiments (data not shown).

Also, we did not see any significant difference between germline cells and Hey cells in the *in vitro* NER activity (data not shown). Interestingly, a significant difference was observed in the expression of EGFR between germline tumour cells and ovarian cancer (Hey) cells, while the expression of JNK1 and JNK2 showed no difference between them (Figure 2).

### Expression of EGFR enhances the drug resistance of germline cells

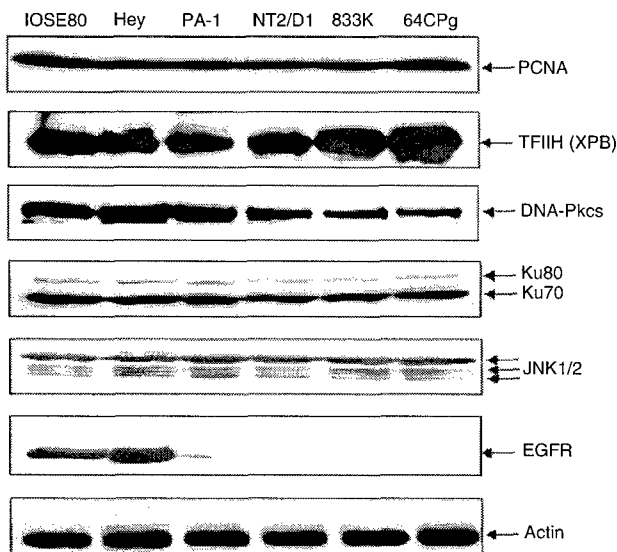
To further examine whether the lack (or low level) of EGFR expression in germline tumour cells (Figure 3A) contributes to their drug sensitivity, cells were transfected with plasmid DNA expressing either green fluorescence protein (GFP) or GFP-EGFR fusion protein and analysed for their effect on drug resistance of cells. After initial selection of cells expressing GFP or GFP-EGFR, protein expression and cellular localization were analysed by Western blot (Figure 3B) and by fluorescence microscopy (Figure 3C), respectively. Germline tumour cells harbouring pEGFR-GFP plasmid showed a high level of EGFR expression, which was comparable to that in drug-resistant ovarian cancer (Hey) cells (Figure 3B). Cells harbouring pEGFR-GFP not pEGFP-N3 showed EGF-dependent activation of JNK1, suggesting that GFP-EGFR fusion protein is functionally active (data not shown).

Germline cells transfected with pEGFR-GFP showed only a marginal increase in their cell survival following cisplatin treatment, while cells expressing GFP (pEGFP-N3) exhibited a slight decline in cell survival (Figure 4 and Table 1). When a stably transfected cell instead of transient system was examined for drug sensitivity, however, it not only showed a significant increase in EGFR expression, but also enhanced survival of germline tumour cells following cisplatin treatment (Figure 5 and Table 1). Although EGFR kinase is activated by EGF, we did not see a substantial increase in cell survival in the presence of EGF probably because EGFR can also be activated by cisplatin. The difference in cell survival between transiently transfected cells (Figure 4) vs stable transfectants (Figure 5) following drug treatment may be due to the lower transfection efficiency in transient system, where only 30% of cells expressed GFP-EGFR (data not shown). Together, our results suggest that (1) a lack (or lower level) of EGFR expression



**Figure 1** Effect of various drugs on the survival of GCTs (PA-1, 833K, NT2/D1, and 64CP9), ovarian primary epithelial cells (IOSE 80), and ovarian cancer cells (Hey). Cells were treated for 72 h with various concentrations of cisplatin (A), MMC (B), and adriamycin (C). Percentage of surviving cells was monitored using MTT assay and the results are the averages of three independent assays.

in germline tumour cells contributes to their drug sensitivity and (2) EGFR may play a positive role in protecting cells following treatment of cells with DNA-damaging agent.



**Figure 2** Expression of various proteins in germline cells. Extracts (100 µg) from various germline tumour cells (PA-1, 833K, NT2/D1, and 64CP9) and ovarian cells (Hey and IOSE-80) were analysed for the expression of DNA repair factors or damage signalling proteins by Western blot.

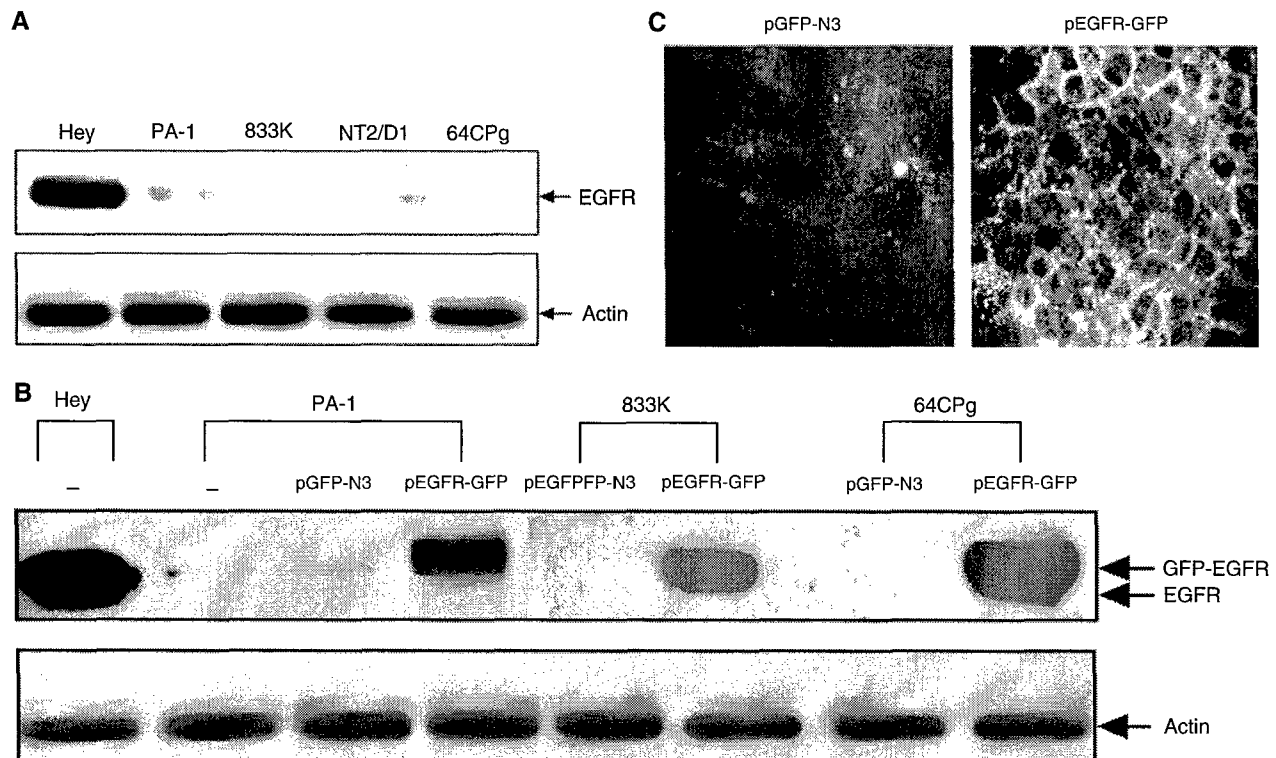
### Lack (or lower level) of EGFR expression in primary germline cells

To see whether lack or lower level of EGFR expression is a common property of germline tumour cells, a number of primary GCTs were selected and tested for EGFR expression. Among 61 GCTs tested, 35 showed undetectable level of EGFR expression, while the remaining samples expressed very low level of EGFR compared to a control ovarian cancer cells (Table 2), supporting the observation with established cells (PA-1, NT2/D1, 833K, and 64CP9) that germline tumours express lower level of EGFR (Figure 3). In fact, the probability of all 61 GCT samples having EGFR expression no higher than + is extremely low ( $2 \times 10^{-25}$ ).

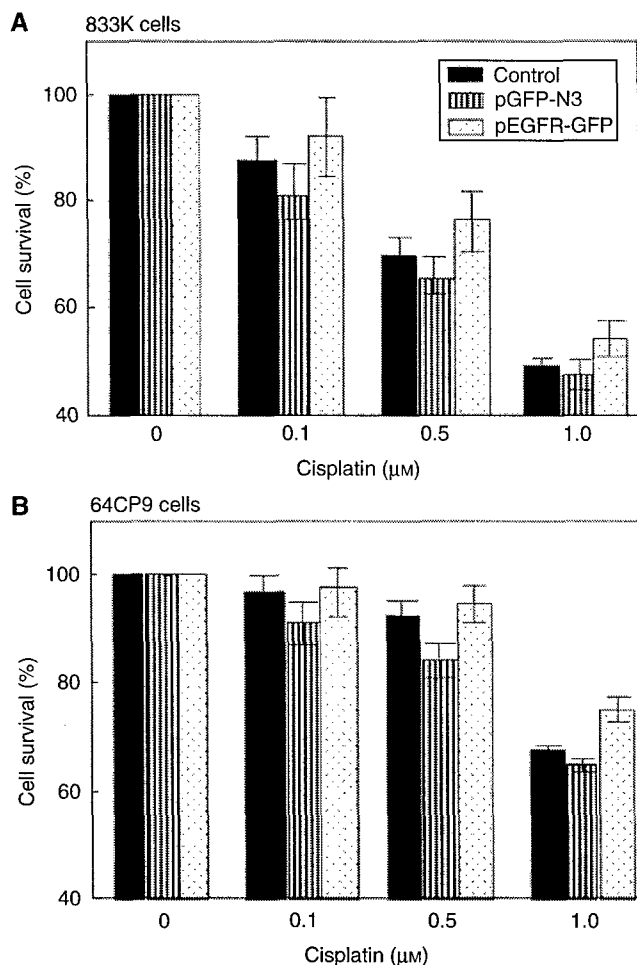
### DISCUSSION

Alteration of DNA repair factors or damage response proteins has been associated with drug resistance of cancer cells (Mohrenweiser *et al*, 2003). For example, a tumour suppressor gene, p53, is a key DNA damage mediator that plays a dual role following exposure to cytotoxic treatment (Ferrera *et al*, 1999); it is involved in damage-induced apoptosis, but also plays a role in cell cycle arrest and DNA repair, cellular processes that can affect the sensitivity to chemotherapeutic drug. However, a consensus on the role for DNA repair genes in drug resistance of various cancer cells has not been reached, mainly because the complicated nature of drug-induced resistance with various tumours made it difficult to delineate a single mechanism (such as DNA repair) that contributes to the resistance.

Compared to drug-resistant ovarian cancer cells, germline tumour cells showed a marked sensitivity following the treatment



**Figure 3** Whole-cell lysates (30 µg) from various germline tumour cells (PA-1, 833K, NT2/D1, and 64CP9) and Hey cells were examined for the expression of EGFR by Western blot (A). In (B), PA-1 cell lines were stably transfected with either pEGFP-N3 vector or pEGFR-GFP, while 833K and 64CP9 cells were transiently transfected for 36 h with pEGFP-N3 or pEGFR-GFP (see 'Materials and Methods' for the details). Expression of EGFR-GFP was monitored by Western blot. (C) shows the expression of GFP (left) or GFP-EGFR (right) in PA-1 cells that were stably transfected with pEGFP-N3 or pEGFR-GFP, respectively. For immunofluorescence, cells were fixed and permeabilised briefly with methanol incubated with anti-EGFR antibody as described under 'Materials and Methods'.



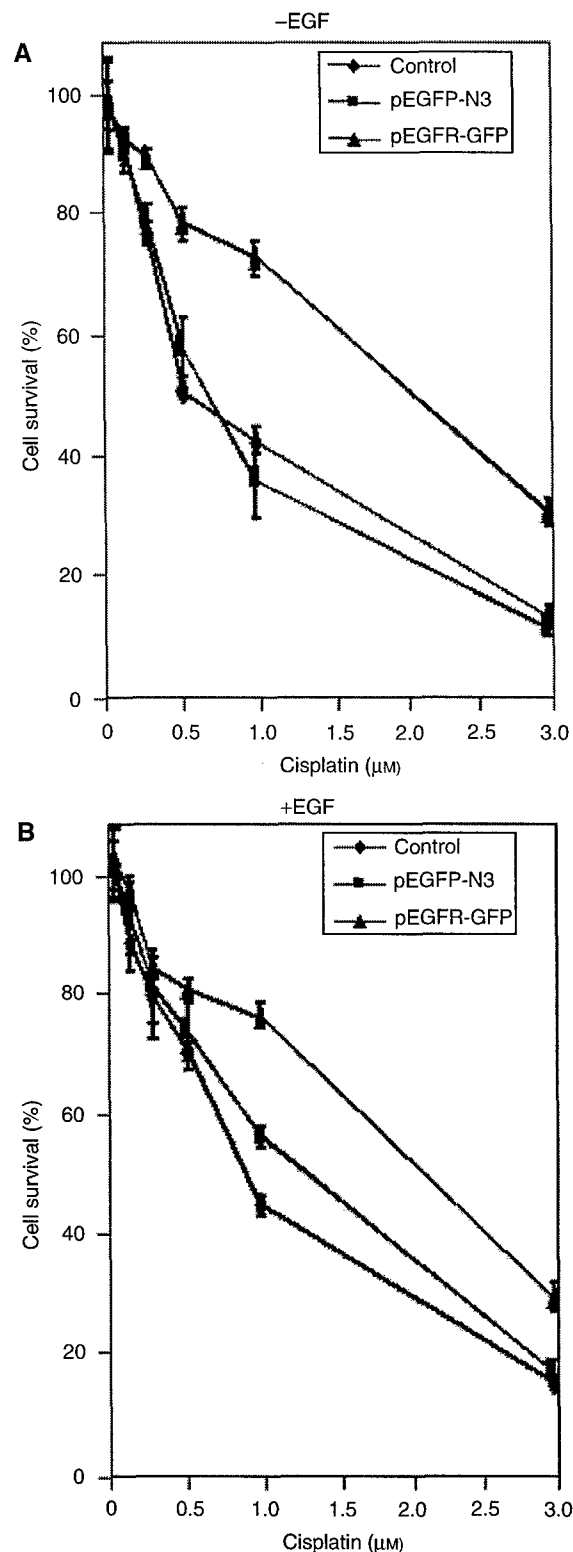
**Figure 4** Transient expression of EGFR enhances the survival of two germline tumour cells (833K (**A**); 64CP9 (**B**)) following cisplatin treatment. Control cells were compared with those transiently transfected with either pEGFP-N3 vector or pEGFR-GFP and examined for their cell survival following cisplatin treatment. At 24 h after transfection, cells were exposed to the indicated amount of cisplatin for 72 h. The percentage of surviving cells was monitored by MTT assay.

**Table 1** Effect of EGFR expression on cisplatin resistance of germline tumour cells (833K) following cisplatin treatment

Cisplatin ( $\mu\text{M}$ )	Mean cell survival rate (%)		
	pEGFP-N3	pEGFR-GFP	P-value from t-test
(-EGF)			
0.1	81.25	95.5	0.006
0.5	69.25	78	0.008
1.0	49.25	57.25	0.002
(+EGF)			
0.1	93.25	98	0.047
0.5	87.75	97.5	0.001
1.0	64.25	75.25	0.004

Cells expressing EGFR (pEGFR-GFP) were compared with control cells (pEGFP-N3) for cell survival following cisplatin treatment in the presence and absence of EGF ( $n = 4$ ).

with cisplatin, adriamycin, or MMC (Figure 1). Examination of the established cell lines as well as primary germcell tumours for genetic alteration of several key repair factors and damage signalling factors indicated that drug sensitivity of germline



**Figure 5** Overexpression of EGFR markedly increased the survival of PA-I cells following cisplatin treatment. Control cells (PA-I) were compared with those stably transfected with either pEGFP-N3 vector or pEGFR-GFP for their cell survival in the presence (**A**) and absence (**B**) of EGF following cisplatin treatment. At 24 h after the seeding, cells were exposed to the indicated amount of cisplatin for 7 days before counting colonies ( $> 50$  cells colony $^{-1}$ ). Each point is the mean value of triplicate experiments.

**Table 2** Germ cell tumour (GCT) samples scored for EGFR expression by immunohistochemistry

GCT#	EGFR	GCT#	EGFR	GCT#	EGFR
LSAB	++++	GCT 1218	+	GCT 1191	-
GC control	+	GCT 1108-2	+	GCT 1171	-
GCT 1221	+	GCT 1131-2	-	GCT 1169	-
GCT 1130-2	+	GCT 1132	-	GCT 1190	-
GCT 1115	-	GCT 1142	+	GCT 1183	-
GCT 1230-2	+	GCT 1116	-	GCT 1182	-
GCT 1206	+	GCT 1124	-	GCT 1181	+
GCT 1110	+	GCT 1127	-	GCT 1156	+
GCT 1208	+	GCT 1118	-	GCT 1122	+
GCT 1106	-	GCT 1229	-	GCT 1199	-
GCT 1108	+	GCT 1144	-	GCT 1198	-
GCT 1135	-	GCT 1202	+	GCT 1160	+
GCT 1143-2	-	GCT 1154	-	GCT 1204	-
GCT 1134	-	GCT 1153	-	GCT 1224	+
GCT 1215	-	GCT 1151	-	GCT 1158	+
GCT 1182-2	+	GCT 1150	-		
GCT 1212	+	GCT 1160-2	-		
GCT 1145-2	+	GCT 1147	+		
GCT 1125	+	GCT 1161-2	-		
GCT 1097-2	+	GCT 2470-9	-		
GCT 1098	+	GCT 1165	-		
GCT 1100	+	GCT 1166	-		
GCT 1138	-	GCT 1168	-		
GCT 1214	-	GCT 1194	-		

Two control cells (ovarian cancer cells (Hey, LSAB) and a normal germ cell, GC control) were included to compare the level of EGFR expression in GCT. '-' represents no detectable EGFR expression, while the level of protein expression was determined by comparing with two control cells, GC control at the lowest level (+) and LSAB at the highest level (++++).

tumour cells may not be due to an alteration of repair factors or DNA repair activity (Figure 2). Instead, there was a good correlation between EGFR expression (or EGF-induced JNK activation) and drug resistance among ovarian and germline tumour cells. Low level of EGFR expression in germline tumour cells may be linked to their drug sensitivity and supports a positive role for EGFR in drug resistance of cancer. The latter may be explained by the fact that EGF and its receptor activate the JNK signalling pathway that leads to the induction of genes involved in DNA repair and cellular redox (Adler *et al*, 1992; Foltz *et al*, 1998; Roulston *et al*, 1998).

Epidermal growth factor receptor is a 170 kDa transmembrane glycoprotein with tyrosine kinase activity. Although EGFR was shown to have no independent prognostic significance in advanced cancer (Baekelandt *et al*, 1999), the EGFR and HER2/neu were frequently overexpressed in malignant tumours. Recent micro-assay analysis revealed that amplification of EGFR gene was found in many tumours including ovarian cancer (Lei *et al*, 1999), glioblastoma (Hui *et al*, 2001), pancreatic cancer (Bruell *et al*, 2003; Schreiner *et al*, 2003), gastric cancer (Garcia *et al*, 2003), prostate cancer (Skacel *et al*, 2001), and lung adenocarcinoma and head/

neck squamous cell carcinoma (Haedicke *et al*, 2003; Shintani *et al*, 2003), suggesting that overexpression of EGFR may be linked to the oncogenesis of various cancers. High level of EGFR expression also correlates with increased tumour resistance to radiation (Shintani *et al*, 2003), suggesting that EGFR may mediate radioresistance of cancer cells (Liang *et al*, 2003). Epidermal growth factor receptor is also a cellular receptor for human cytomegalovirus, a cancer-causing virus that causes severe and fatal disease in immune-compromised individuals (Wang *et al*, 2003).

Epidermal growth factor receptor-associated protein tyrosine kinase complexes also have vital antiapoptotic functions in human breast cancers (Modjtahedi *et al*, 1998; Witters *et al*, 1999) and the blockade of EGFR not only adversely affected cell growth, but also showed a sign of terminal differentiation and induces apoptosis in the human cancer cells (Modjtahedi *et al*, 1998). Similarly, drug-induced apoptosis in human breast cancer cells was abrogated by using EGFR antisense RNA (Dixit *et al*, 1997), suggesting that a critical level of EGFR signalling, which is amplified in some common cancers, may be necessary for DNA-damaging drug-mediated apoptosis in tumour cells and suggest an inhibitory effect of this pathway on the repair of cisplatin-damaged DNA. In fact, cancer cells expressing higher levels of EGFR were much more resistant to the growth inhibitory effect of DNA-damaging agents than were control cells (Dixit *et al*, 1997).

Various strategies have been developed to target EGFR and to deter cancer cell growth (Zhang *et al*, 2000; Bruell *et al*, 2003; Heimberger *et al*, 2003). For example, the treatment of cancer cells with EGFR tyrosine kinase inhibitor markedly potentiates the efficacy of many cytotoxic agents against several human cancer xenografts (She *et al*, 2003). The use of antisense oligonucleotides or monoclonal antibodies to EGFR also showed significant inhibition of cancer cell growth (Modjtahedi *et al*, 1998; Witters *et al*, 1999), while activation of EGFR family members suppresses the cytotoxic effects of TNF-alpha (Hoffmann *et al*, 1998).

Although mutations in proto-oncogenes (*c-ret*) as well as DNA MMR genes have been linked to germline tumours (van Puijenbroek *et al*, 1997; Leung *et al*, 2000), alteration of EGFR in germcell tumours has not been reported. This study showed that germline tumour cells not only exhibited lower EGFR expression but also were highly sensitive to DNA-damaging drugs, suggesting that the lack of EGFR expression contributes at least in part to the drug sensitivity of germline cells.

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## Forum Review

### The Intracellular Localization of APE1/Ref-1: More than a Passive Phenomenon?

GIANLUCA TELL,<sup>1</sup> GIUSEPPE DAMANTE,<sup>1</sup> DAVID CALDWELL,<sup>2</sup> and MARK R. KELLEY<sup>2</sup>

#### ABSTRACT

Human apurinic/aprimidinic endonuclease 1/redox effector factor-1 (APE1/Ref-1) is a perfect paradigm of the functional complexity of a biological macromolecule. First, it plays a crucial role, by both redox-dependent and -independent mechanisms, as a transcriptional coactivator for different transcription factors, either ubiquitous (*i.e.*, AP-1, Egr-1, NF- $\kappa$ B, p53, HIF) or tissue-specific (*i.e.*, PEBP-2, Pax-5 and -8, TTF-1), in controlling different cellular processes such as apoptosis, proliferation, and differentiation. Second, it acts, as an apurinic/aprimidinic endonuclease, during the second step of the DNA base excision repair pathway, which is responsible for the repair of cellular alkylation and oxidative DNA damages. Third, it controls the intracellular reactive oxygen species production by negatively regulating the activity of the Ras-related GTPase Rac1. Despite these known functions of APE1/Ref-1, information is still scanty about the molecular mechanisms responsible for the coordinated control of its several activities. Some evidence suggests that the expression and subcellular localization of APE1/Ref-1 are finely tuned. APE1/Ref-1 is a ubiquitous protein, but its expression pattern differs according to the different cell types. APE1/Ref-1 subcellular localization is mainly nuclear, but cytoplasmic staining has also been reported, the latter being associated with mitochondria and/or presence within the endoplasmic reticulum. It is not by chance that both expression and subcellular localization are altered in several metabolic and proliferative disorders, such as in tumors and aging. Moreover, a fundamental role played by different posttranslational modifications in modulating APE1/Ref-1 functional activity is becoming evident. In the present review, we tried to put together a growing body of information concerning APE1/Ref-1's different functions, shedding new light on present and future directions to understand fully this unique molecule. *Antioxid. Redox Signal.* 7, xxx-xxx.

#### INTRODUCTION

##### *General considerations on APE1/Ref-1 biological and molecular functions*

**A**PE1/REF-1 is a perfect example of the functional complexity of a biological macromolecule. Its acronym reflects its at least dual nature: human apurinic/aprimidinic (AP) endonuclease, or APE1 (also HAP1 or APEX), is a major constituent of the base excision repair (BER) pathway

of DNA lesions. Ref-1, the acronym for redox effector factor-1, refers to its redox abilities on different redox-regulated transcription factors (TFs). Interestingly, these two activities are split into two functionally independent domains of the protein itself: the N-terminus is principally devoted to the redox activity, whereas the C-terminus exerts enzymatic activity on the abasic sites of DNA (135). Different from the N-terminus, which is completely unconserved, the C-terminus is highly conserved from plant to man.

Both of these two activities seem to be fundamental in the control of the apoptotic process, as demonstrated by several

<sup>1</sup>Department of Biomedical Sciences and Technologies, University of Udine, Udine, Italy.

<sup>2</sup>Department of Pediatrics, Herman B. Wells Center for Pediatric Research and the Departments of Biochemistry & Molecular Biology and Pharmacology & Toxicology, Indiana University School of Medicine, Indianapolis, IN.

works (14, 96, 126). APE1/Ref-1 expression is always inversely correlated with the onset of apoptosis, suggesting a role as an antiapoptotic molecule.

### DNA-repair activity of APE1/Ref-1

APE1/Ref-1 is an essential protein (136) that contributes, through its participation in the BER pathways, to the regeneration of DNA damaged by products of cell metabolism and by environmental hazards. The two best characterized functions of APE1/Ref-1 in these pathways are production of a DNA primer for repair synthesis and coordination of the repair activities of other BER proteins (21, 28, 47, 63, 104, 129).

BER repairs DNA damage with a set of enzyme activities that sequentially remove the damaged base (glycosylases), incise the phosphodiester backbone 5' adjacent to the abasic site (APE1/Ref-1), excise the abasic residue [APE1/Ref-1,  $\beta$  polymerase, or flap endonuclease 1 (FEN1)], polymerize the replacement nucleotide(s) [ $\beta$  polymerase, x-ray cross-species complementing 1 (XRCC1), or  $\delta/\epsilon$  polymerases with proliferating cell nuclear antigen (PCNA)], and ligate the final sequence (DNA ligases I and III, XRCC1) (Fig. 1).

BER repair synthesis requires a DNA primer with a 3' hydroxyl end. APE1/Ref-1 generates this end in three ways. It binds specifically (102) and processively (13) to abasic sites, generated spontaneously or by glycosylases, and cuts the 5' phosphodiester bond with its endonuclease activity to produce the primer. This endonuclease activity depends on the redox state of APE1/Ref-1, controlled in part by amino acid C310 (70). APE1/Ref-1 removes with its 3'-5' exonuclease activity a 3'-phospho- $\alpha,\beta$ -unsaturated aldehyde, formed by complex glycosylases [such as the oxidative damage-repairing glycosylases 8-oxoguanine DNA glycosylase (Ogg1) and Nth] and by radiation. This activity may also contribute to the fidelity of repair synthesis by removing mispaired nucleotides (132). And, although less efficiently than PNK, APE1/Ref-1 removes with its 3' phosphatase activity a 3' terminal phosphate, produced by glycosylases NEIL1 and NEIL2 and by radiation (63). The repair function of APE1/Ref-1 requires 10 evolutionarily conserved amino acids, D70, D90, E96, Y171, D210, N212, D219, D283, D308, and H309 (35).

Coordination of these steps within the BER pathway (106) and with other DNA repair pathways is thought to be important for preventing the accumulation of toxic repair intermediates (105, 131) (each a type of DNA damage) and for increasing the overall efficiency of the pathways. Abasic sites produced spontaneously or by glycosylases can inhibit DNA and RNA polymerases, facilitate mutation, and promote single-strand breaks (129, 145). Single-strand breaks produced by complex glycosylases and by APE1/Ref-1 can inhibit polymerases, promote recombination, and become double-strand breaks during replication, which in turn can lead to chromosome rearrangements and cell death (11). Evidence from reconstituted systems and from cell extracts suggests that APE1/Ref-1 contributes to coordination by interacting directly or indirectly with other BER enzymes and with other repair pathways. The molecular basis of the specificity and of the stimulatory mechanisms and the biological significance of these interactions remain to be determined.

The glycosylase MYH (MutY DNA glycosylase homologue) removes adenine and 2-hydroxyadenine mismatched with guanine or 7,8-dihydro-8-oxodeoxyguanine (89, 90). APE1/Ref-1 increases MYH activity 10-fold in a reconstituted system by associating directly with MYH to promote the formation of efficient MYH-DNA complexes by decreasing the MYH-DNA substrate dissociation constant (142). Immunoprecipitation and affinity chromatography show that this association requires MYH amino acids 295-317 containing the conserved motif S/PGXZDV/I, where X and Z are any amino acids (89). APE1/Ref-1 may also bind directly to methylpurine glycosylase, as suggested by *in vitro* binding studies and by far western analysis of a nuclear ~30-kDa protein with APE1/Ref-1 in CHO cell extracts (35), although yeast two-hybrid analysis failed to detect this interaction (5).

Other glycosylases appear to interact with APE1/Ref-1 indirectly, through competition for DNA binding sites. Glycosylases Ogg1 (54), Nth1 (74), uracil DNA glycosylase (88), and thymine DNA glycosylase (128) display product inhibition, binding tightly to their processed DNA product. This binding may help recruit APE1/Ref-1 to the damaged site and protect the AP sites or single-strand breaks until APE1/Ref-1 continues the repair process. APE1/Ref-1 alleviates this inhibition by displacing the glycosylases through its stronger association with the DNA.

Yeast two-hybrid and gel supershift analyses suggest that a direct interaction between DNA-bound APE1/Ref-1 and DNA polymerase  $\beta$  (5) recruits polymerase  $\beta$  to the damaged site, stimulating fivefold the rate of removal of the abasic sugar (5' dRp) by the lyase activity of polymerase  $\beta$ . This stimulation and consequent repair synthesis in turn increase APE1/Ref-1 endonuclease activity, by removing through repair synthesis the 3' terminus that effects product inhibition of APE1/Ref-1 (77).

In addition to its role in single-strand break repair, XRCC1 may act as a scaffold to physically organize the BER process. XRCC1 binds directly to APE1/Ref-1 in yeast two-hybrid, far western, and affinity chromatography assays (122). This interaction requires the N-terminus of APE1/Ref-1, stimulates the rate of APE1/Ref-1 endonuclease activity fivefold, and stimulates the 3' phosphodiesterase activity. Extracts prepared from CHO cells defective in XRCC1 activity show diminished APE1/Ref-1 endonuclease activity that is rescued by addition of XRCC1. Stimulation of this endonuclease activity may also decrease the lyase activity of Ogg1 to increase the efficiency of repair (75).

Coprecipitation and affinity chromatography show that PCNA, the sliding clamp processivity factor of DNA polymerases  $\delta$  and  $\epsilon$ , and FEN1, the flap endonuclease of long-patch BER, bind directly to APE1/Ref-1 (22). APE1/Ref-1 stimulates flap excision twofold in a reconstituted system, suggesting the APE1/Ref-1 interaction with FEN1 may be functional. Tom *et al.* (118) have suggested that the ability of APE1/Ref-1 to organize BER proteins in the presence and absence of PCNA may contribute to the differential regulation of BER and DNA replication during oxidative stress mediated by p21.

p53 binds directly to APE1/Ref-1 and stimulates BER, although the relationship of the binding and stimulation is unknown and p53 is not required for BER (105). Far western analysis of purified proteins and immunoprecipitation west-

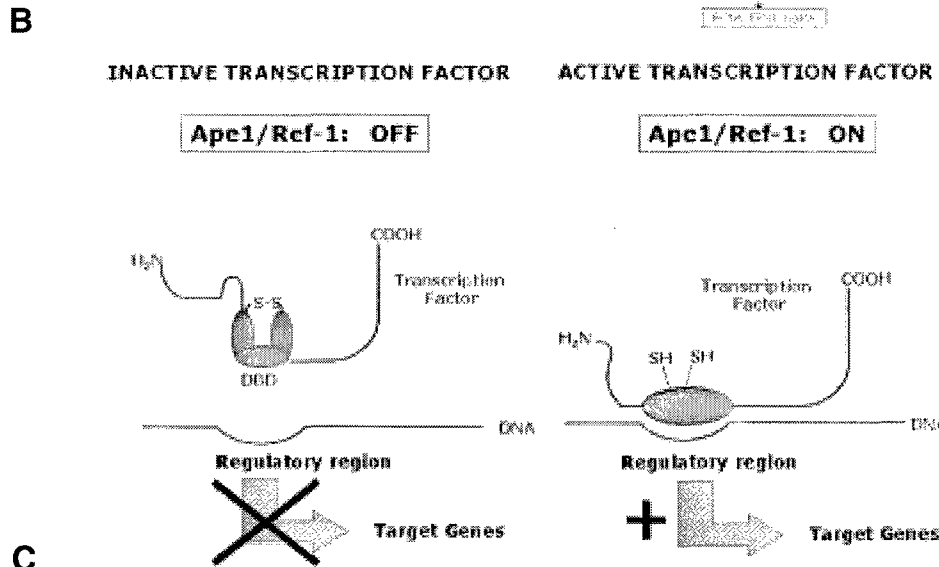
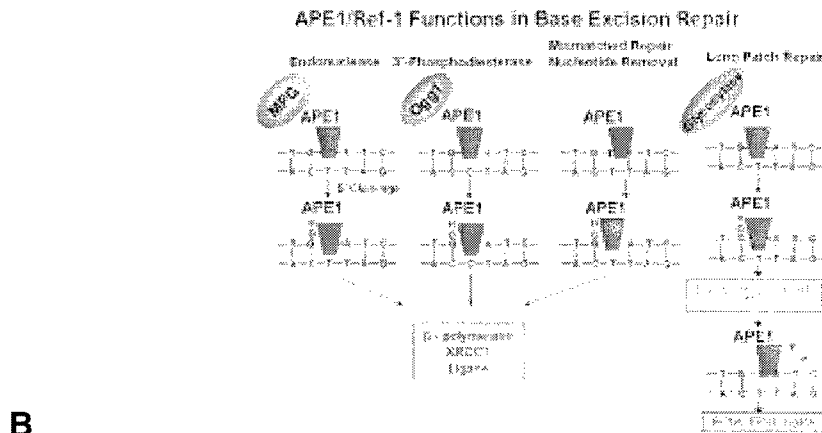
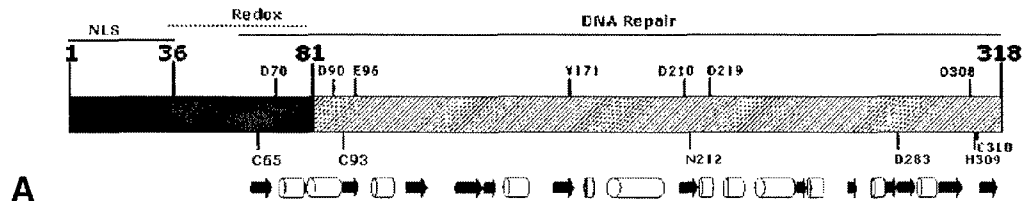
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**FIG. 1. Representations of APE1/Ref-1 structure and functions.** (A) Schematic structure of APE1/Ref-1 with critical residues. NLS, nuclear localization signal. Cylinders represent  $\alpha$ -helical regions, and arrows represent  $\beta$ -strands, as deduced by Gorman *et al.* (44). (B) APE1/Ref-1 functions in BER. MPG, methylpurine DNA glycosylase; RFC, replication factor C. (C) Theoretical molecular model of the redox function of APE1/Ref-1 as a transcriptional coactivator. DBD, DNA binding domain.

ern analysis of H24-14 cell extracts show that the tumor suppressor p53 binds directly to APE1/Ref-1 (41). p53 also stimulates BER in nuclear extracts and in reconstituted systems, dependent on its N-terminal transactivating region (146). Equal APE1/Ref-1-specific activities in extracts prepared from isogenic cell lines with wild-type and suppressed p53

suggest that the stimulation is not due to interaction between p53 and APE1/Ref-1 (101).

APE1/Ref-1 protein interactions may also influence DNA repair pathways other than BER. Affinity chromatography and gel mobility shift assays show that the single-strand break repair proteins Ku 70/80 bind APE1/Ref-1 (16).

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Whether this interaction affects either BER or single-strand break repair is unknown.

### Redox regulation of TFs activities

Redox regulation of cellular functions occurs as a consequence of the so-called "redox-cellular status," which is the result of a balance between the activity of antioxidant enzymatic cell systems (such as GSH/GSSG, superoxide dismutase, catalase, peroxidases, glutathione peroxidases, etc.) and the amount of reactive oxygen species (ROS) such as superoxide anion ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radical ( $\cdot OH$ ). These last molecules can be produced in several ways: as by-products of respiration, thus being associated with cell proliferation rate; by external noxious agents, such as ionizing radiation (127); during pathological states in activated neutrophils (80); and as "second messengers" produced by intracellular enzymatic systems, such as NADPH oxidase regulated by the ubiquitous small GTPase Rac1 (20, 24, 43). It therefore represents a useful tuning device for intracellular signal transduction, as is the case in cascades induced by cytokines, such as tumor necrosis factor- $\alpha$  or interleukin (IL)- $\beta$  (80).

This redox regulation ultimately affects gene expression. Recently, a great body of experimental evidence suggested that these outcomes are achieved through modulation of TFs activity. Up to now, several TFs containing specific Cys residues have been demonstrated to be the target of redox regulation. APE1/Ref-1 has been identified as a protein capable

of nuclear redox activity, inducing the DNA-binding activity of several TFs, such as AP-1 (133), NF- $\kappa B$  (83), Myb (134), PEBP-2 (1), HLF (27), NF-Y (81), Egr-1 (60), HIF-1 $\alpha$  (59), ATF/CREB family (134), p53 (41), Pax proteins (12, 110, 112). It accomplishes this through the control of the redox state of Cys residues located in the DNA-binding domains or within regulatory regions, such as the transactivation domain of the thyroid-specific transcription factor 1 (*i.e.*, TTF-1) of the TFs themselves (115). In order to properly bind specific DNA target sequences, these TFs require that critical Cys residues are in the reduced state. Therefore, by maintaining these cysteines in the reduced state, APE1/Ref-1 provides a redox-dependent mechanism for regulation of target gene expression. APE1/Ref-1 contains two cysteine residues located within the redox-active domain (Cys65 and Cys93), and previous studies show that Cys65 should be the redox-active site of the protein by using recombinant protein (123). In agreement with the molecular model describing redox regulation exerted by APE1/Ref-1, Cys65 should interact with the sensitive cysteine residues within the DNA-binding domains of TFs. However, Jayaraman *et al.* (64) suggest that the stimulatory role played by APE1/Ref-1 on p53 activity may also occur in a redox-independent way. This has been recently corroborated by the work of Ordway *et al.* (85) in which the authors provide first *in vivo* evidence that the Cys65 residue of APE1/Ref-1 is, unexpectedly, not essential for redox regulation of AP-1 DNA binding. However, these authors did not completely exclude a possible presence of compensatory phe-

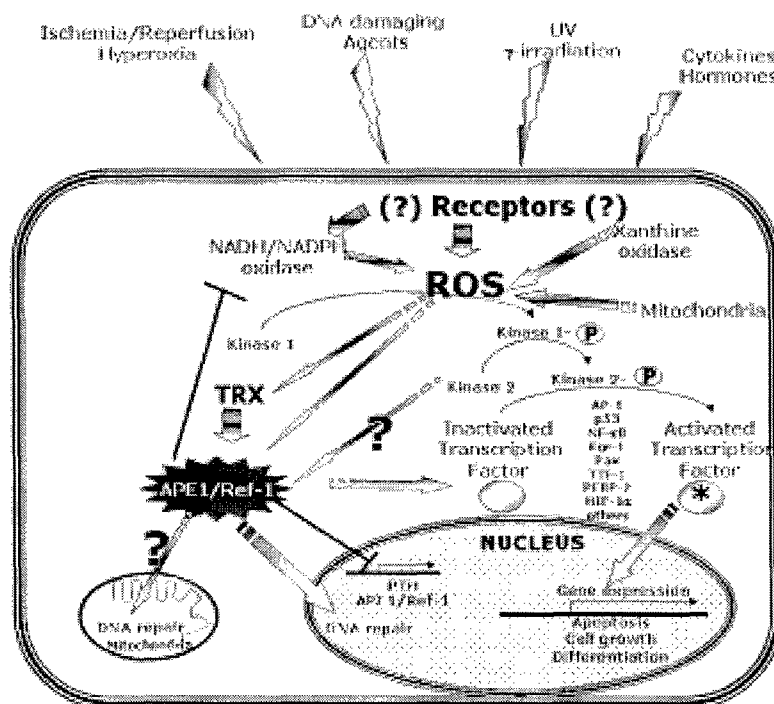


FIG. 2. Schematic representation of some of the stimuli known to activate APE1/Ref-1 expression and/or function.

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nomena. In any case, this evidence challenges previous hypotheses about the molecular mechanisms by which APE1/Ref-1 exerts its redox-dependent activities of specific TFs.

According to the proposed redox-regulatory role on cellular functions played by APE1/Ref-1, both gene expression and protein levels are up-regulated by nontoxic levels of a variety of ROS and/or ROS-generating systems (Fig. 2).

### REGULATION OF APE1/REF-1 FUNCTIONS: AT THE GENE EXPRESSION LEVEL AND POSTTRANSLATIONAL (PT) MODIFICATIONS

Early evidence of APE1/Ref-1 regulation came from expression studies. It was immediately clear that APE1/Ref-1 expression was principally controlled at the transcriptional level (Table 1). Several studies, both *in vivo* and *in vitro*, clearly demonstrated that different oxidative agents efficiently and quickly (within minutes to hours) promote APE1/Ref-1 transient increase at both the mRNA and protein levels (Fig. 2). As it is blocked by cycloheximide, the latter process requires *de novo* protein synthesis (94, 112). Up-regulation of APE1/Ref-1 protein levels appears to have biological relevance. In fact, protein up-regulation is always associated with an increase in both redox and AP endonuclease activity, followed by an increase in cell resistance toward oxidative stress and DNA-damaging agents (46, 94). Therefore, an important issue was the identification of extracellular signals able to increase APE1/Ref-1 gene expression.

As experimental data grew, so did research aimed at identifying biological modulators of APE1/Ref-1 gene expression, such as hormones and cytokines. With respect to these observations, recent articles by our group (113, 116) together with that of Asai *et al.* (3) depicted a clear view of the thyrotropin (TSH)-induced APE1/Ref-1 gene expression in thyroid cells. It became immediately clear that functional triggering of membrane-bound receptors could be responsible for a positive regulation of APE1/Ref-1 gene expression itself for other cell systems. Such an example is represented by the human chorionic gonadotropin that has been previously demonstrated to induce APE1/Ref-1 mRNA synthesis in murine Leydig cells (107). In the case of the immune system, a physiological induction of APE1/Ref-1 gene expression has been recently demonstrated for at least two cell types. This is the case of human alveolar macrophages stimulated with granulocyte-monocyte colony-stimulating factor (31), which can be released by these cells during fibrotic processes in the lung, and of spleen B cells stimulated with CD40 ligand (78). In the first case, a functional role in AP-1 transcriptional activity has been proposed. In the latter case, the CD40 triggering is functional to the activation of *blk* promoter operated by Pax5a and EBF TFs. A role for IL-2-stimulated APE1/Ref-1 up-regulation has also been demonstrated in a murine pro-B cell line (140). Very recently, a functional role for this process has been suggested involving redox regulation of telomerase activity (137).

Other soluble mediators that have been investigated dealing with APE1/Ref-1 expression are dopamine and glutare-

doxin (Grx2) in cerebellar granule neurons. Daily *et al.* (17) demonstrated that the endogenous neurotransmitter of the nigrostriatal pathway, *i.e.*, the proapoptotic dopamine, exerts an inhibitory effect on APE1/Ref-1 expression that is squelched by the antioxidant Grx2, thus leading to NF- $\kappa$ B activation and to cell protection from apoptosis.

APE1/Ref-1 levels seem to act as an intracellular signaling device. In fact, its protein levels correlate with the propensity of the cell to undergo apoptosis or proliferation. In practice, modulation of APE1/Ref-1 protein expression has been described for almost every cell type depending on the particular cellular redox status induced by exposure to an oxidative environment (Table 1). Moreover, physiological stimuli, such as those of cytokines, are able to promote APE1/Ref-1 up-regulation. Indeed, cell systems must be able to discriminate these different stimuli if APE1/Ref-1 behaves as a signaling molecule.

At present, it is not known what the *primum movens* responsible for APE1/Ref-1 gene expression upon oxidative stress is or the signaling pathways leading to its activation. A major role seems to be represented by cyclic AMP as a common second messenger in the case of both TSH and H<sub>2</sub>O<sub>2</sub> stimulations (3, 116). However, from the comparative study of cytokine- and ROS-induced APE1/Ref-1 up-regulation, some conclusions could be drawn about the molecular switches responsible for its modulated expression. Moreover, as APE1/Ref-1 expression represents a molecular marker of oxidative stress, it would be important to understand the dissection of signaling pathways responsible for its role in regulating the mechanisms of ROS-induced cell responses.

The human *APE1/REF-1* gene is located on chromosome 14q11.2–12 and span 2.6 kb, and consists of four introns and five exons, the first of which is noncoding. Its *cis*-regulatory regions are composed of a proximal basal promoter of ~300 bp containing a CpG island (Fig. 3A). Within this area, several putative binding sites for redox-regulated (such as AP-1, Sp-1, and ATF) or cyclic AMP-regulated (such as CREB) transcriptional factors are present. At the distal promoter level, at least three negative calcium-responsive regulatory elements (nCaREs) were found: nCaRE-A, nCaRE-B1, and nCaRE-B2 (62). By binding to one of these regulatory elements (nCaRE-B2), APE1/Ref-1 is able to repress its own expression, thus providing a means to control its intracellular levels (62). Therefore, APE1/Ref-1 can also exert transcriptional control by binding to specific DNA sequences in cooperation with hnRNP-L, a member of the heterogeneous ribonucleoproteins (73). This has also been confirmed by findings demonstrating the ability of APE1/Ref-1 to repress the parathyroid hormone (PTH) gene expression (84). In fact, PTH promoter is characterized by the presence of a nCaRE binding motif that is specifically recognized by APE1/Ref-1.

Despite the fact that the APE1/Ref-1 gene was cloned several years ago (50) and its promoter characterized in detail (52), very few articles have addressed the molecular mechanisms responsible for its inducible regulation. Up to now, the unique ascertained contributors to APE1/Ref-1 transcriptional activation are that of Jun/ATF2 in an inducible way in response to oxidative stress (45) and that of Sp-1 required for both the basal (region -65 to -17 from the transcription start site) and the coordinate (region +99 to +183) expression of

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TABLE I. SUMMARY OF STUDIES DESIGNED TO INDUCE APE/REF-1 AT THE MRNA AND/OR THE PROTEIN LEVELS

<i>Tissue/cells</i>	<i>Treatment or pathological condition</i>	<i>Gene expression</i>	<i>Subcellular compartment</i>	<i>Reference</i>	<i>Proposed biological role</i>
HT-29	Hypoxia	↑		144	Involvement in detoxification of xenobiotics
HeLa	UV and hypoxia	↑		124	Protection against hypoxic stress
Rat dentate gyrus (granular cells)	Ischemia	↑	N	42	Neuronal protection against oxidative stress
Rat hippocampus (CA1 neurons)	Ischemia	↑	N	42	Neuronal protection against oxidative stress
	Hypoxic-ischemic insult	↓		125	Development of apoptosis
CHO	ROS	↑		46	Clastogenic adaptive response to oxidative stress
HeLa S3 and W138	ROS	↑		94	Adaptive response to ROS causing enhanced repair of cytotoxic DNA lesions
Raji (B lymphocytes)	ROS	↑	N	112	Redox regulation of TFs activity
		↑	M	36	DNA repair
Rat hippocampus	Ischemia	↓		26	Development of apoptosis
Rat mesothelial cells	Asbestos	↑		39	DNA repair
FRTL-5 (rat thyroid cells)	TSH, ROS, elevation in intracellular Ca <sup>2+</sup>	↑	N	3, 113, 116	Redox regulation of TFs activity
Rat SON and PVN cells	Hypertonic	=		95	DNA repair
Rat SCN cells	Light	=		95	DNA repair
Porcine epidermis	Wound healing	↑		51	DNA repair
MA-10	hCG	↑		107	Redox regulation of TFs activity
Liver	Ischemia/reperfusion	↑		87	Redox regulation of TFs activity, Control of intracellular ROS production
CPAEC and HUVEC	Hypoxia	↓		49	Redox regulation of TFs activity
RBL-2H3 mast cells	ROS	↑	N	37	Redox regulation of TFs activity
Spleen B cells	CD40 triggering	↑	N	78	Redox regulation of TFs activity
HT29 colon adenocarcinoma cells	Dithiolethione oltipraz	↑		143	Redox regulation of TFs activity
Alzheimer's disease	Chronic ROS	↑		19	DNA repair
Rat kidney	Aging	↑	N	15	Redox regulation of TFs activity
Human choriocarcinoma cells	Elevation in intracellular Ca <sup>2+</sup>	↑	N	38	Transcriptional repression
Rat liver treated with WY14,643	ROS	↑	M	56	DNA repair
Rabbit spinal cord	Ischemia	↓		99	DNA repair, development of apoptosis
Human atherosclerotic plaques	ROS	↑		76	DNA repair
Human fibroblasts	Arsenic	↑		58	Redox regulation of TFs activity
HUVEC	Hypoxia/reoxygenation	↑	N	2	Redox regulation of TFs activity
Human macrophages	Asbestos	↑	N	32	Redox regulation of TFs activity
Human alveolar macrophages	GM-CSF	↑		31	Redox regulation of TFs activity
K562 human myeloid cell line	PMA, hypochlorite, MMS	↑		57	Redox regulation of TFs activity, DNA repair
BA/F3 murine pro-B cell line	IL-2	↑		140	Redox regulation of TFs activity
				137	Stimulation of telomerase activity

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TABLE 1. (CONTINUED)

Tissue/cells	Treatment or pathological condition	Gene expression	Subcellular compartment	Reference	Proposed biological role
Murine cerebellar granule neurons	Dopamine	↓		17	Redox regulation of TFs activity
Murine cerebellar granule neurons	GRx2	↑	N/C	18	Redox regulation of TFs activity, protection from apoptosis
Astrocyte primary cultures	Pb	↑	N	100	Redox regulation of TFs activity
CHO	ROS	↑		45	Redox regulation of TFs activity
Rat tamoxifen-induced hepatocarcinogenesis	Tamoxifen	↑		69	Redox regulation of TFs activity, DNA repair
Rat duodenal mucosa	Cysteamine-induced duodenal ulceration	↑	N	71	Redox regulation of TFs activity
Human placenta	Preeclampsia	↑		108	DNA repair

C, cytoplasm; CPAEC, calf pulmonary artery endothelial cells; GM-CSF, granulocyte-monocyte colony-stimulating factor; hCG, human chorionic gonadotropin; HUVEC, human umbilical vein endothelial cells; M, mitochondria; MMS, methyl methanesulfonate; N, nucleus; PMA, phorbol 12-myristate 13-acetate; PVN, paraventricular nuclei; SCN, suprachiasmatic nuclei; SON, supraoptic nuclei.

APE1/Ref-1 during cell cycle (40). These last results were obtained in the case of the mouse promoter. However, as a conserved Sp-1 binding site located downstream from the +1 transcription start site is present in the human promoter (region about +76 to +85), a similar behavior could be expected. Very recently, an important role in the control of APE1/Ref-1 gene expression has also been suggested for the signal transducer and activator of transcription-3 (Stat3) TF in liver (48), suggesting a new mechanism of protection against Fas-mediated liver injury. These findings confirm that much work remains to be done in order to elucidate, in detail, the molecular mechanisms of controlling APE1/Ref-1 gene expression.

Due to the fact that recombinant APE1/Ref-1 expressed in *E. coli* fully retains functional redox and endonuclease activity, it was erroneously thought for several years that APE1/Ref-1 was not subjected to PT modifications. The only functional exception to perfectly similar behavior between endogenous and recombinant APE1/Ref-1 was represented by a differential binding activity toward the nCaRE sites. In fact, recombinant APE1/Ref-1 alone was not able to bind to both nCaRE-A and nCaRE-B in the PTH gene promoter, indicating a requirement for additional factors in the complex (16, 73). These observations led to the identification of Ku70 (Ku86) and hnRNP-L involvement in the complex formation with nCaRE-A and nCaRE-B, respectively. Moreover, APE1/Ref-1 is an abundant protein in a eukaryotic cell. We have estimated that ~10–30,000 copies of the protein are present in a thyroid cell. Therefore, it is possible that many proteins for many different functions have to be “finely tuned” in order to coordinate specific biological activities. The best choice for a biological system to “recycle” the same protein for different biological activities is by means of PT modifications. First, *in silico* studies soon discovered that several different phosphorylation sites were scattered throughout the molecule. These potential phosphorylation sites included consensus sequences for casein kinase I and II (CKI and CKII), for protein kinase

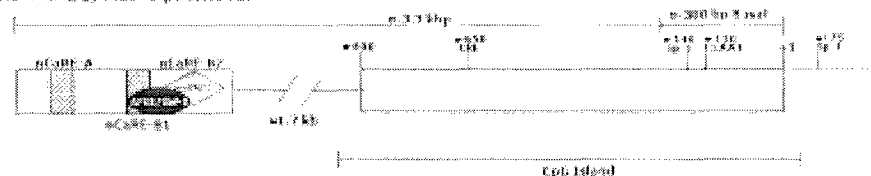
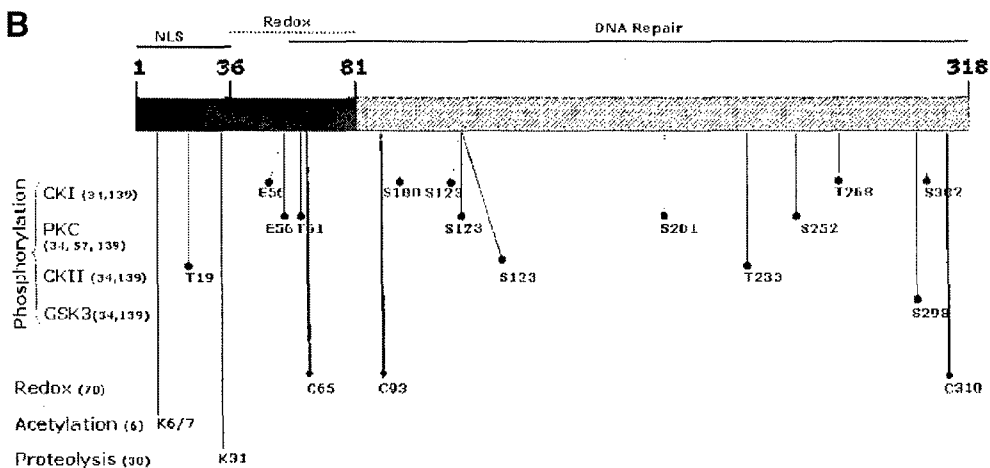
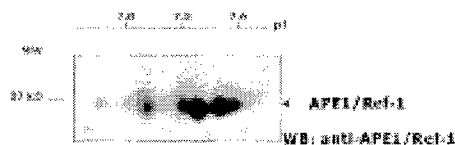
C (PKC), and for glycogen synthase kinase 3 (GSK3) (Fig. 3B) (34, 139). These two pioneering studies were, however, in disagreement regarding the potential phosphorylation sites determined and the functional consequences of *in vitro* phosphorylation.

These first studies were performed *in vitro* by using the recombinant APE1/Ref-1 protein expressed in *E. coli*. The first *in vivo* study was that of Hsieh *et al.* (57), which demonstrated for the first time the occurrence of a PKC-mediated phosphorylation on APE1/Ref-1 protein. Unfortunately, these authors did not determine the location of the specific phosphorylation site. The PKC-mediated phosphorylation was able to promote the redox activity of APE1/Ref-1 on AP-1 TF in response to phorbol 12-myristate 13-acetate treatment or to the oxidizing agent hypochlorite followed by methyl methane sulfonate treatment. However, this study did not address the question of whether endonuclease activity of APE1/Ref-1 is affected by PKC phosphorylation.

Another PT modification that was initially suspected and finally demonstrated *in vitro* was redox modification. Initial evidence came from studies of association with the dithiol-reducing enzyme thioredoxin (TRX) (55, 93, 121). Although the specific residues of APE1/Ref-1 involved in interaction with TRX are not known, Cys35 and Cys32 in the catalytic center of TRX have been demonstrated to be involved. As it was previously demonstrated that Cys65 and Cys93 of APE1/Ref-1 are redox-sensitive (123, 124, 135), it could be speculated that target residues of TRX interaction may include these two cysteine residues. This TRX-mediated redox regulation of APE1/Ref-1 is required for p53 and AP-1 functional activation (55, 121). Very recently, Kelley and Parsons (70) demonstrated that the repair activity is also redox-regulated with the specific involvement of C310 residue located immediately adjacent to the crucial histidine residue at position 309 within the DNA repair active site. Interestingly, oxidation occurring at other Cys residues located in the redox domain

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**A** A.1 Human APE1/Ref-1 gene structure, Chromosome 14q11.2-12: 5 exons and 4 introns**A.2** Human APE1/Ref-1 promoter**B****C****RAT FRTL-5 THYROID CELLS****HUMAN HOBIT OSTEOBLASTIC CELLS**

**FIG. 3. Regulation of APE/Ref-1 functions: at the gene expression level and PT modifications.** (A) Schematic structure of the APE1/Ref-1 gene (A.1) and regulatory region on its promoter (A.2), which is located approximately within ~3 kb upstream of the transcription start site. (B) Schematic diagram of putative or demonstrated PT modifications of APE1/Ref-1. (C) *In vivo* heterogeneity of APE1/Ref-1 protein associated with different PT modifications. Two-dimensional gel electrophoresis (2-DE) of 50  $\mu$ g of nuclear extracts from (upper) rat FRTL-5 thyroid cells and (lower) HOBIT human osteoblastic cell line. After isoelectric focusing on an Immobiline dry strip (pH gradient 6–11) in an IPhor electrophoresis system (Amersham Biosciences), the proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10%) and APE1/Ref-1 was visualized by western blotting by using a specific rabbit polyclonal antibody (114).

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does not affect the repair activity, confirming the functional independence of the two domains of APE1/Ref-1. The structural and functional modularity of APE1/Ref-1, together with this new evidence of different sensitivity toward different specific PT modifications, could explain the fine-tuning required for proper function of the protein.

PT regulation of APE1/Ref-1 protein is responsible for enhanced cell death mediated by granzyme A (GzmA) (30) in K562 cells. APE1/Ref-1 is associated with the endoplasmic reticulum (ER) in a macromolecular complex of 270–420 kDa containing evolutionarily conserved proteins called SET, pp32, and HMG2. GzmA cleaves APE1/Ref-1 after Lys31 and destroys its known DNA repair functions, forcing the cell to undergo apoptosis. These recently published data suggest that a complex array of interrelationships may control APE1/Ref-1 function.

Lastly, acetylation occurring on APE1/Ref-1 protein has very recently been found by Bhakat *et al.* (6). The transcriptional coactivator p300, which is activated by  $Ca^{2+}$ , is able to specifically acetylate APE1/Ref-1 both *in vitro* and *in vivo*. Acetylation at Lys6 or Lys7 enhances DNA binding of APE1/Ref-1 to nCaRE sequences, thus unraveling a means to down-regulate the PTH promoter by APE1/Ref-1 itself. These authors showed that APE1/Ref-1 protein is heterogeneously PT modified in HeLa cells used in this study. Interestingly, and more generally, this heterogeneity is not restricted to the cell line used in this work because our studies, performed on rat FRTL-5 cells and human HOBIT osteoblastic cells by using two-dimensional gel electrophoresis (2-DE) analysis coupled to western blot identification, confirmed that APE1/Ref-1 is heterogeneously present in cell nuclear extracts at basal conditions (see Fig. 3C).

Together, these recent observations have raised the possibility that subtle PT modifications provide a means for channeling the multifunctional APE1/Ref-1 to different activities and interactions and thus could act as a regulatory switch in performing different functions.

## REGULATION OF APE1/REF-1 FUNCTIONS: AT THE SUBCELLULAR LOCALIZATION LEVEL

Most reports have APE1/Ref-1 localized to the nucleus, but a growing body of evidence has shown that in some cell types, particularly those with high metabolic or proliferative rates, such as spermatocytes, thyrocytes, lymphocytes, hepatocytes, and hippocampal cells, APE1/Ref-1 can be cytoplasmic (25, 65, 67, 95, 112, 113, 130). Recent advances provided at least two functional explanations for the cytoplasmic expression of APE1/Ref-1. Previous evidence comes from our work on mitochondrial localization of APE1/Ref-1 (114), confirming previous findings by Fung *et al.* (39) and Tomkinson *et al.* (119). Due to occurrence of oxidative phosphorylation, mitochondria produce a large amount of ROS. Mitochondrial DNA (mtDNA), being located in the mitochondrial matrix, is extremely susceptible to oxidative damage, which represents a major mutational drive for mtDNA itself. The ex-

istence of DNA repair devices in mitochondria is still up for debate (8). However, the presence of the major abasic endonuclease repair enzyme APE1/Ref-1 in this subcellular compartment would account for the existence of a BER mechanism in mitochondria. The latter functional explanation of APE1/Ref-1 cytoplasmic location is related to its association with ER membranes. By using electron microscopy immunocytochemistry, we evidenced this association in FRTL-5 thyroid cells (114). However, more functional information about ER localization of APE1/Ref-1 protein came from recent work by Fan *et al.* (29, 30). APE1/Ref-1 associates with the tumor suppressor protein pp32 and with the nucleosome assembly protein SET in the so-called SET complex in K562 and HeLa cells. Within this context, APE1/Ref-1 together with the HMG2 architectural TF was demonstrated to be a physiologically relevant GzmA substrate in targeted cells. In so doing, GzmA may block cellular repair and force cells into apoptosis.

Moreover, cytoplasmic localization may be required in order to maintain newly synthesized TF in a reduced state while they are being translocated to the nucleus. In this respect, Duguid *et al.* (25) demonstrated an association of APE1/Ref-1 with ribosomes in the cytoplasm of hypoglossal neurons.

Another level of complexity in understanding the regulation/meaning of APE1/Ref-1 biological functions is represented by the widespread observation that the subcellular distribution of this protein is somewhat heterogeneous within cell subpopulations. This is the case of cerebellar granule cells. Their APE1/Ref-1 staining is mostly cytoplasmic, whereas adjacent cells show intense nuclear staining (67). The recent observation that APE1/Ref-1 is subjected to a cell cycle-dependent expression (40) could explain this. These data, paired with the demonstration of cell cycle-dependent hMYH glycosylase expression (9), suggest that coordinated expression of key DNA repair genes with the cell cycle is a general phenomenon. Moreover, as APE1/Ref-1 plays an important role in contributing to p53-mediated cell-cycle arrest (41) by regulating p53 TF activity through both redox-dependent and -independent mechanisms (64), and it is down-regulated during apoptosis in myeloid leukemia cells (96), then it is possible that the role of cell cycle-responsive APE1/Ref-1 expression relates to one of these other functions rather than to DNA repair.

Finally, in many cell types, APE1/Ref-1 displays both nuclear and cytoplasmic localization. These cell types include normal thyroid cells stimulated by TSH, transformed thyroid cells (113), mucosal and parietal cells of the stomach, cerebellar Purkinje cells, adrenal cortical cells, and some cervical cells (67, 95).

Therefore, to sum up, APE1/Ref-1 subcellular localization is quite varied. Some cell types exhibit only nuclear localization, others display only cytoplasmic, and some others show both nuclear and cytoplasmic localization. Such a complex staining pattern suggests that localization is not random but, on the contrary, is governed by a strictly regulated process. The understanding of the biological relevance of subcellular compartmentalization is now at its very beginning, but recent articles suggest that this field shows much promise.

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### APE1/REF-1 FUNCTIONS, SUBCELLULAR MOVEMENT, AND TRAFFICKING

Generally, stimuli that promote APE1/Ref-1 expression are also able to promote its intracellular movement. The amount of evidence reporting APE1/Ref-1 subcellular relocalization upon a stimulus has grown exponentially in the last few years and is shown in Table 2. Most of the observed subcellular relocalizations are cytoplasm-to-nucleus APE1/Ref-1 translocations. Different cellular conditions are able to perturb the APE1/Ref-1 intracellular localization. Five categories of cellular stimuli can be identified: (a) prooxidant injuries, such as neuronal ischemia (42), hypoxia/reoxygenation in endothelial cells (2), cysteamine-induced duodenal ulceration (71), and aging (15); (b) heavy metals exposure, such as macrophages exposed to asbestos (32), and primary astrocyte cultures exposed to Pb (100); (c) direct ROS exposure, as in the case of B-lymphocytes (36, 112) or mast cells (37); (d) hormone or cytokine stimulation as in the case of TSH-stimulated thyroid cells (113), or CD40-triggering in splenic B-cells (78) and FcεRI signaling in mast cells (37); and (e) increase in intracellular  $Ca^{2+}$  concentration in both choriodecidual cells (38) and thyroid cells (116).

The question of how eukaryotic cells regulate the localization of APE1/Ref-1 with regard to the redox condition remains open. In many cases, it has been demonstrated that PT modifications, *i.e.*, phosphorylation at the level of nuclear localization signal (NLS), are responsible for the regulation of the protein levels present in the nuclear compartment. Computational analysis reveals that APE1/Ref-1 possesses a single consensus sequence for CKII at position 18–21, a region containing a NLS (TEPE). In particular, treatment of FRTL-5 thyroid cells with quercetin, a highly specific CKII inhibitor,

is able to induce translocation of APE1/Ref-1 into the nucleus (unpublished observations), suggesting that the nuclear form of APE1 could be hypophosphorylated. Therefore, it is tempting to speculate that a dephosphorylating event is required for the nuclear localization of APE1/Ref-1 in thyroid cells, as in the case of the NF-AT TF (103).

As APE1/Ref-1 directly and physically interacts with redox-regulated TFs, this raises the possibility that nuclear relocalization could be, at least in part, due to a co-transportation mechanism by those TFs, such as NF-κB, that are specifically relocalized to the nucleus due to a stimulus and have been demonstrated to functionally interact with APE1/Ref-1 itself. This would not represent an isolated case of such a mechanism, being corroborated by recent reports for a role in cotransport of β-catenin by the LEF-1 TF (61).

Another interesting evidence is the case of the observed relocalization of APE1/Ref-1 into mitochondria of the B-lymphocyte Raji cell line following  $H_2O_2$  activation (36). This relocalization is not associated with cytochrome *c* loss or with apoptosis induction, thus indicating that the APE1/Ref-1 translocation into mitochondria upon oxidative stress might exert a protective function toward mtDNA damages produced by both exogenous and endogenous ROS. Due to the relatively high molecular weight of APE1/Ref-1, which is not fully compatible with a passive mechanism of translocation through the outer membrane of mitochondria, the presence of a specific regulatory transport mechanism could be hypothesized. Although a large majority of proteins synthesized in the cytoplasm localize into mitochondria by means of an N-terminal presequence, or mitochondrial targeting sequence (MTS), a significant fraction of mitochondrial proteins lack this recognition signal. Little is known about other mitochondrial targeting signals, but there could be targeting signals within the molecule itself. This is the situation, for instance,

TABLE 2. SUMMARY OF STUDIES DESIGNED TO INDUCE APE1/REF-1 SUBCELLULAR RELOCALIZATION

<i>Tissue/cells</i>	<i>Treatment or pathological condition</i>	<i>Subcellular compartment</i>	<i>Translocation</i>	<i>Reference</i>	<i>Proposed biological role</i>
Rat hippocampus (CA1 neurons)	Ischemia	N	+	42	Neuronal protection against oxidative stress
Raji (B lymphocytes)	ROS	N	+	112	Redox regulation of TFs activity
		M	+	36	DNA repair
FRTL-5 (rat thyroid cells)	TSH, ROS, elevation in intracellular $Ca^{2+}$	N	+	113, 116	Redox regulation of TFs activity
Spleen B cells	CD40 triggering	N	+	78	Redox regulation of TFs activity
Rat kidney	Aging	N	+	15	Redox regulation of TFs activity
Human choriodecidual cells	Elevation in intracellular $Ca^{2+}$	N	+	38	Transcriptional repression
HUVEC	Hypoxia/reoxygenation	N	+	2	Redox regulation of TFs activity
Human macrophages	Asbestos	N	+	32	Redox regulation of TFs activity
Astrocyte primary cultures	Pb	N	+	100	Redox regulation of TFs activity
Rat duodenal mucosa	Cysteamine-induced duodenal ulceration	N	+	71	Redox regulation of TFs activity
RBL-2H3 mast cells	ROS and FcεRI-signalling	N	+	37	Redox regulation of TFs activity

HUVEC, human umbilical vein endothelial cells; M, mitochondria; N, nucleus.

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in the heme lyases that have, in the third quarter of the molecule, a hydrophilic stretch of residues that represent a topogenic signal for protein sorting into the intermembrane space (23). APE1/Ref-1 does not present a classical mitochondrial N-terminal presequence while presenting, in the same region, a typical NLS in the first 20 residues. Therefore, it could be speculated that the mitochondrial localization signal may reside in another region of the molecule instead of the N-terminus. Obviously, identification of such a signal would represent an important discovery that would elucidate some of the functional roles of APE1/Ref-1. Recently, Tsuchimoto *et al.* (120) demonstrated the presence of MTS in APE2, which is a classical mammalian AP endonuclease enzyme belonging to the same functional category as APE1/Ref-1. As opposed to APE1/Ref-1 and other members of this functional category, the presence of a 15-stretch amino acid N-terminal sequence resembling a canonical MTS is responsible for the localization of the protein within mitochondria. As in the case of APE1/Ref-1, APE2 is also localized within the nucleus of HeLa cells. Despite this evidence and opposed to APE1/Ref-1, APE2 does not possess a classical NLS that can justify nuclear localization. However, this subcellular localization seems to be ascribable to the presence of a PCNA binding motif in the C-terminus of the protein through which it physically interacts with PCNA itself.

A recent role that has been proposed for the cytoplasmic APE1/Ref-1 came from studies on endothelial and liver oxidative stress by hypoxia/reoxygenation-induced oxidative stress (2, 87). From these studies, a new role for APE1/Ref-1 in protecting cells from apoptosis induced by oxidative stress was suggested. In fact, APE1/Ref-1 is able to inhibit oxidative stress by inhibiting ROS generation by the cytoplasmic small GTPase Rac1, therefore accounting for a new and unexplored extranuclear role of APE1/Ref-1.

## ALTERED EXPRESSION/DISTRIBUTION OF APE1/REF-1 AND HUMAN PATHOLOGY

The multifunctional properties of APE1/Ref-1 closely parallel the differential expression pattern found in a wide spectrum of cells first realized by Kakolyris *et al.* in 1998 (67). In the following years, many articles observed that this heterogeneity of expression pattern is also linked to different pathological conditions ranging from metabolic to differentiative disorders. Early studies by Kakolyris *et al.* showed that different kinds of human tumors were characterized by alterations in subcellular distribution of APE1/Ref-1 with respect to normal tissue. This is the case, for instance, in colorectal carcinoma. In normal colorectal mucosa, the predominant staining is nuclear in the less differentiated cells of the lower part of the crypt, but is cytoplasmic in the more differentiated and superficial colonic epithelium. This distribution is completely disrupted during tumorigenesis because the nuclear-restricted pattern is lost in both adenoma and carcinoma, which display nuclear and cytoplasmic localization with a predomination of the latter, in front of a prominent nuclear localization in the normal tissue (65). A similar pattern has been described for breast cancer. In normal tissue, the

APE1/Ref-1 localization is eminently nuclear, whereas in carcinomas, nuclear, cytoplasmic, and nuclear/cytoplasmic stainings were observed. This peculiar distribution correlates well with aggressiveness and prognosis of the tumor: nuclear localization was always associated with a better prognostic feature being related to better differentiation, low angiogenesis, and negative lymph node status. In contrast, both the cytoplasmic and the nuclear/cytoplasmic stainings were associated with poor prognostic factors, such as angiogenesis together with node and p53 positivities (66, 92). Interestingly, there seems to be no functional relationship between alterations in subcellular distribution of APE1/Ref-1 and the ability of cancerous tissue to repair abasic sites, suggesting that, at least in breast cancer, DNA repair by BER is not affected (98). As an aside, Bobola *et al.* (7) demonstrated that even an increase in AP endonucleasic activity occurs in human gliomagenesis with a concomitant elevation of APE1/Ref-1 protein expression level. Similar observations have also been made by Robertson *et al.* (97). In different germ cell tumors, such as seminomas, yolk sac tumors, and malignant teratomas, APE1/Ref-1 expression levels and DNA repair ability correlated and conferred a proportional level of protection from bleomycin treatment. These outcomes suggest that a consequence of the increase in AP activity accompanying tumorigenesis could be enhanced resistance to radiotherapy and chemotherapy.

A dysregulation in nuclear versus cytoplasmic ratio toward a more cytoplasmic staining was also observed in thyroid carcinomas (113) and epithelial ovarian cancers (79) with respect to normal tissue.

In other studies, a quantitative evaluation of APE1/Ref-1 expression was taken into consideration. In cervical (138), non-small cell lung cancer (68, 91), rhabdomyosarcomas (117), and squamous cell head-and-neck cancer (72), a strong up-regulation at the nuclear level of APE1/Ref-1 was always observed. Some studies suggested that APE1/Ref-1 levels and/or subcellular dysregulation may be used as a therapeutic index to indicate the sensitivity of the tumor toward radio- or chemotherapy. Koukourakis *et al.* (72) found that nuclear expression of APE1/Ref-1 in head-and-neck cancer was associated with resistance to cisplatin chemoradiation therapy and poor outcome. Herring *et al.* (53) reported the existence of an inverse relationship between intrinsic radiosensitivity and the levels of APE1/Ref-1 in cervical carcinoma. However, these are not general features of all tumors. In fact, although it has been clearly demonstrated that a subcellular dysregulation was associated with the onset of the tumorigenic process in ovarian cancer (79), APE1/Ref-1 expression was ubiquitous between different epithelial ovarian cancers and was unaltered during the metastatic process (33). Moreover, APE1/Ref-1 proved not to be a useful biomarker for platinum resistance, because there was no difference in its expression among platinum-sensitive and platinum-refractory ovarian cancers. These recent outcomes highlight the fact that much work needs to be done in order to understand the real possibility of using APE1/Ref-1 as a therapeutic target.

Probably, the right direction in which to investigate the role played by APE1/Ref-1 in tumorigenesis is that indicated by recent results on PT modifications of APE1/Ref-1 itself. In particular, Orii *et al.* (86) investigated the role played by

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APE1/Ref-1 in uterine leiomyomas; the most common benign smooth muscle tumors in the myometrium, by evaluating its expression at both the mRNA and protein levels. Despite the presence of a unique mRNA transcript, these authors were able to detect at least two forms of the protein by using a combination of three antibodies raised against different epitopes of the protein itself. The large form of the protein, prominent in leiomyoma extracts with respect to myometrial tissue extracts, was correlated with PCNA levels, suggesting an association with increased proliferation (86). These observations on PT modifications of APE1/Ref-1, coupled with those cited in the previous paragraph on acetylation and phosphorylation, confirm a new investigative direction for the comprehension of APE1/Ref-1 multifaceted function and for its therapeutic potential.

Cellular oxidative stress is a common pathogenic event in different disorders, therefore, it was expected that APE1/Ref-1 expression and/or subcellular localization could be affected in those pathologies where oxidative stress is a shared feature. Other than in proliferative disorders mentioned above, APE1/Ref-1 dysregulation has also been demonstrated for other pathologies, particularly degenerative disorders. Gillardon *et al.* (42) observed that transient global ischemia induced by cardiac arrest was able to activate both APE1/Ref-1 mRNA expression and protein nuclear accumulation in granular cells of the ischemia-resistant dentate gyrus at 6 h after injury. In contrast, these effects were absent in CA1 pyramidal neurons of the posts ischemic hippocampus. Moreover, the decrease in APE1/Ref-1 protein, but not in its mRNA, was followed by an increase in the apoptotic rate, suggesting the presence of an opposite relationship existing among APE1/Ref-1 expression and apoptosis. The lack of correspondence between mRNA and protein levels emphasizes the importance of studying protein PT modifications to understand the functional roles and regulation of APE1/Ref-1 protein. Evidence published by Gillardon *et al.* (42) suggests that, in the case of CA1 pyramidal neurons, these discrepancies could be due to the presence of a posttranscriptional block during the translation process or to an increased degradation of the protein upon ischemia. Interestingly, it was also demonstrated that APE1/Ref-1 may undergo ubiquitination by Ubc9 enzyme (141). A similar decrease of APE1/Ref-1 protein levels was recently observed in motor neurons in a rabbit model of spinal cord ischemia by Sakurai *et al.* (99). It is noteworthy that this reduction preceded oxidative DNA damage and may constitute one of the factors responsible for the delay in neuronal death after spinal cord ischemia. It is probably not casual that cells characterized by a high rate of metabolic activities, such as neurons, are also vulnerable when DNA repair or redox controlling systems are defective. In fact, these cells are a particular target of the noxious effects of ROS at both the DNA and protein levels. Another important neurological disorder with which APE1/Ref-1 has been associated is Alzheimer's disease. Hippocampi of affected patients show an increase in APE1/Ref-1 levels in senile plaques and plaque-like structures (109). This evidence accounts for a wider involvement of the DNA repair mechanisms in Alzheimer's disease, as recently suggested by Davydov *et al.* (19).

Recent evidence also points to a role of APE1/Ref-1 in cardiovascular disease. Analysis of endomyocardial biopsies

from patients with dilated cardiomyopathy demonstrated an association between left ventricular wall stress and up-regulation of APE1/Ref-1 expression associated with active DNA repair (4). Blood vessels are also subjected to disorders with an oxidative stress-based etiopathogenesis. In fact, ROS formation is a crucial event in atherosclerosis because it causes oxidation of low-density lipoprotein, hyperproliferation, and, when damage is excessive, apoptosis. In this light, it is not surprising that several DNA repair systems involved in BER, such as APE1/Ref-1, are associated with this pathology (76).

Finally, two other pathologies in which APE1/Ref-1 alteration is involved are inclusion body myositis (IBM) and preeclampsia. In the first case, APE1/Ref-1 localized in paired-helical filaments, amyloid-like fibrils, and amorphous material in muscle of IBM patients suggested a role played by APE1/Ref-1 in IBM pathogenesis (10). In the second case, an up-regulation of APE1/Ref-1 and TRX was associated with preeclampsia and intrauterine growth restriction in complicated pregnancies, definitively suggesting that cellular redox regulation is affected in the pathophysiology of this disorder (108).

## CONCLUDING REMARKS AND PERSPECTIVES

APE1/Ref-1 is a perfect paradigm of the functional complexity of a biological macromolecule. First, as a transcriptional coactivator, it impacts on a wide variety of cellular functions ranging from the control of proliferation to apoptosis, cytokines, and hormone signaling where ROS are mainly produced and function as second messengers. Second, as a DNA-repair enzyme, it plays a fundamental role in the BER pathways of DNA lesions caused by oxidants and/or alkylating agents. Therefore, as APE1/Ref-1 is a master player of the above-mentioned cellular decisions, it is not surprising that (a) it is critical to the survival of animals (136); (b) it is ubiquitously expressed in every cell type, and (c) generally, it is quite abundantly expressed. However, for a coordinated and strictly integrated functioning of APE1/Ref-1 in the proper biological context, it is necessary that its different functions must be highly regulated. It is known that APE1/Ref-1 is an inducible protein whose expression is controlled mainly at the transcriptional level by the action of APE1/Ref-1 itself, which functions as a suppressor of its own promoter activity (62), thus constituting an autoregulatory loop. Moreover, it is coming to light that compartmentalization of the protein in different subcellular districts, such as nucleus, ER, or mitochondria, may explain the different functional specificities. This is particularly reinforced by its complex and heterogeneous staining pattern, which is typical of each cell type, and by the fact that the peculiar subcellular distribution is usually lost during cancer progression, or a cytoplasm to nucleus translocation occurs upon oxidative stimuli in different cell types. All this evidence suggests that subcellular localization and trafficking of APE1/Ref-1 should be highly regulated processes. However, they open another problem by shifting the molecular question to the mechanisms responsible for the control of a so highly regulated process. Hopefully, convinc-

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ing answers will come from the studies on PT modifications occurring on APE1/Ref-1 and responsible for the coordinated control of its different functions. Recent evidence suggests that at least three kinds of PT modifications affect APE1/Ref-1 primary structure, *i.e.*, phosphorylation, redox, and acetylation, and condition its function. This way of looking at the problem of APE1/Ref-1 functioning holds the promise of representing the "Rosetta stone" for the comprehension of this unique biological molecule.

Further improvement in unraveling the "world" of APE1/Ref-1 will also come from new proteomics approaches devoted to the investigation of a biological problem as a whole. The identification of APE1/Ref-1 "interactomes" under different biological conditions will lead to a deeper understanding of the role played by this multifaceted protein in different biological systems. These outcomes will be of help in developing new strategies based on different APE1/Ref-1 functions as therapeutic targets for the several pathologies in which the protein plays a role, such as cancer, neurodegenerative, and immunological disorders. The recent chemogenomic identification of APE1/Ref-1 as a therapeutic target for asthma is suggestive for a promising scenario in the near future (82).

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## ABBREVIATIONS

AP, apurinic/apyrimidinic; AP-1, activator protein-1; APE1, apurinic/apyrimidinic endonuclease 1; BER, base excision repair; CKI and CKII, casein kinase I and II; 2-DE, two-dimensional gel electrophoresis; Egr-1, early growth response protein-1; ER, endoplasmic reticulum; FEN1, flap endonuclease 1; Grx2, glutaredoxin 2; GSK3, glycogen synthase kinase 3; GzmA, granzyme A; HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; HLF, HIF-1 $\alpha$ -like factor; hnRNP-L, heterogeneous nuclear ribonucleoprotein L; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; IBM, inclusion body myositis; IL, interleukin; MTS, mitochondrial targetin sequence; MH, MutY DNA glycosylase homologue; nCaRE, negative calcium-responsive regulatory element; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NLS, nuclear localization signal; Ogg1, 8-oxoguanosine DNA glycosylase; Pax, paired box containing genes; PCNA, proliferating cell nuclear antigen; PEBP-2, polyoma virus enhancer-binding protein-2; PKC, protein kinase C; PT, posttranslational; PTH, parathyroid hormone; Ref-1, redox effector factor-1; ROS, reactive oxygen species; TF, transcription factor; TRX, thio-

redoxin; TSH, thyrotropin; TTF-1, thyroid transcription factor-1; XRCC1, x-ray cross-species complementing 1.

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Address reprint requests to:

Gianluca Tell, M.D.

Department of Biomedical Sciences and Technologies

University of Udine

P. le Kolbe 4

33100 Udine, Italy

E-mail: gtell@mail.dstb.uniud.it

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# **An *in vivo* analysis of MMC-induced DNA damage and its repair**

Young-Ju Lee,<sup>1</sup> Su-Jung Park,<sup>1,3</sup> Samantha L.M. Ciccone,<sup>2</sup> Chong-Rak Kim,<sup>4</sup> and Suk-Hee Lee<sup>1,3\*</sup>

Department of Biochemistry and Molecular Biology<sup>1</sup>, Microbiology<sup>2</sup>, Walther Oncology Center<sup>3</sup>,  
Indiana University School of Medicine, Indianapolis, Indiana 46202; Inje University<sup>4</sup>, Kimhae,  
Korea

Running title: An *in vivo* analysis of the early stage of MMC-induced DNA repair pathways

(Key words: crosslink DNA damage, mitomycin c, psoralen, DNA repair, Xeroderma pigmentosum)

\*Corresponding author: 1044 W. Walnut St. Rm153, Indiana University Cancer Research Institute, Indianapolis, IN 46202. Phone (317) 278-3464; Fax (317) 274-8046; Email: [slee@iupui.edu](mailto:slee@iupui.edu)

## ABSTRACT

Mitomycin (MMC) induces various types of DNA damages that cause a significant cytotoxicity to cells. Accordingly, repair of MMC-induced damages involves multiple repair pathways such as nucleotide-excision repair, homologous recombination repair, and translesion bypass repair pathways. Nonetheless, repair of the MMC-induced DNA damages in mammals have not been fully delineated. In this study, we investigated potential roles for *Xeroderma pigmentosum* (XP) proteins in the repair of MMC-induced DNA damages using an assay that detects the ssDNA patches generated following treatment with MMC or 8-methoxy-psoralen (8-MOP) + UVA. Human wildtype cells formed distinctive ssDNA foci following treatment with MMC or 8-MOP + UVA, but not with those inducing alkylation damage, oxidative damage, or strand-break damage, suggesting that the foci represent ssDNA patches formed during the crosslink repair. In contrast to wildtype cells, mutants defective in XPE and XPG did not form the ssDNA foci following MMC treatment, while XPF mutant cells showed a significantly delayed response in forming the foci. A positive role for XPG in the repair of MMC-induced DNA damages was further supported by observations that cells treated with MMC induced a tight association of XPG with chromatin, and a targeted inhibition of XPG abolished MMC-induced ssDNA foci formation, rendering cells hypersensitive to MMC. Together, our results suggest that XPG along with XPE and XPF play unique role(s) in the repair of MMC-induced DNA damages.

**Abbreviations:** ADR, adriamycin; BrdU, 5'-bromo-2'-deoxyuridine; CDDP, cis dichlorodiammineplatinum(II) (also known as cisplatin); DTT, dithiothreitol; ERCC, excision repair cross-complementing; 8-MOP, 8'-methoxy-psoralen; MMC, Mitomycin C; MMS, methyl methane sulphonate; NER, nucleotide excision repair; PCNA, proliferating cell nuclear antigen; RPA, replication protein A; ssDNA, single-stranded DNA; TCR, transcription-coupled repair; TFII-H, transcription factor II-H; UV, ultraviolet light; XP, xeroderma pigmentosum

## INTRODUCTION

DNA crosslink is a serious damage to chromosomal DNA that blocks key DNA metabolisms including DNA replication and transcription. Therefore, cells' ability to repair crosslink damages is not only crucial for cell survival, but also a determining factor for drug sensitivity of cells. Since crosslink damage involves both strands of DNA, its repair process is likely to occur through the coordinated action of multiple DNA repair pathways such as nucleotide-excision repair, the double-strand break/homologous recombination repair, and/or the translesion bypass pathways [1,2]. A model of crosslink repair has been proposed, in which mammalian cells use excinuclease complex to uncouple the crosslink followed by homologous recombination or a translesion bypass to complete repair of first strand [3,4,5]. These notions are supported by the fact that MMC or other crosslinking agent causes minimal structural distortion on chromosomal DNA and may not be recognized by known damage recognition factors such as XPC-hHR23B, XPA, or RPA [6,7]. Instead, the initiation of crosslink repair is likely coupled to DNA replication or transcription machinery when it stalls at the damaged site [8,9,10].

A homologous recombination-based crosslink repair assay *in vitro* identified a number of proteins involved in ICL repair, including ERCC1-XPF, RPA, PCNA, as well as MutS-beta [11]. RPA was found to be essential for both the formation of incision as well as in the subsequent DNA synthesis step of crosslink damage repair, indicating that it is required for lesion recognition and/or for the subsequent endonucleolytic processing. PCNA is required for the DNA synthesis stage and although it is not critical for the incision stage of the reaction it does enhance this step presumably by a stimulation of lesion recognition by MutSbeta [11]. Loss of bloom syndrome (BLM) gene or fanconi anemia (FA) gene(s) also resulted in hypersensitivity to interstrand crosslinking agent(s),



suggesting that BLM and FA are either directly or indirectly involved in DNA repair or cell survival in response to crosslink damage [10]. A recent study demonstrated that SNM1/PSO2, a gene encoding a 5'-exonuclease, was also required for incision of ICL repair in yeast [12,13]. A siRNA targeting hSNM1B gene rendered cells sensitive to ionizing radiation, suggesting the possibility of hSNM1B involvement in homologous recombination repair of double-strand breaks arising as intermediates of ICL repair [12]. Physical interaction between SNM1A, one of the three SNM1 family in higher eukaryotes, and PIAS1 was required for their nuclear localization as well as for the crosslink repair activity [14].

In an effort to understand the mechanistic details of the ICL repair pathway, we have developed an *in vivo* assay that detects the ssDNA foci generated in response to ICL agents such as MMC or 8'-methoxy-psoralen. WT cells exhibited distinctive ssDNA foci following ICL damages, while cells lacking XPE and XPG did not. On the other hand, XPF mutant cells showed a much delayed in response to ICL damage in forming the ssDNA foci, suggesting that formation of the ssDNA foci likely represents the early stage of ICL repair pathway that requires various XP proteins.

## **MATERIALS AND METHODS**

### **Chemicals and antibodies:**

Mitomycin C (MMC), 5'-bromo-2'-deoxyuridine (BrdU), methyl methane sulphonate (MMS), adriamycin (ADR), cis-dichlorodiammineplatinum(II) (CDDP; also known as cisplatin), H<sub>2</sub>O<sub>2</sub>, and 8'-methoxy-psoralen (8-MOP) were purchased from Sigma Chemical Co. (St. Louis, MO). An anti-BrdU monoclonal antibody specific for BrdU on ssDNA not dsDNA was from the BD Biosciences (San Jose, CA). Anti-XPG (monoclonal), -XPF (monoclonal), and -ERCC1 (monoclonal) antibodies were from the Neo Markers (Fremont, CA). An anti-GFP polyclonal antibody raised against full-length green fluorescent protein (GFP) was from the Santa Cruz Biotechnology (Santa Cruz, CA).

### **Cell lines and their culture conditions:**

Human fibroblasts [GM00037F (WT), GM02415B (XPE), GM08437A (XPF) and GM03021B (XPG)] were grown in minimum essential medium (MEM) supplemented with 20% fetal calf serum (FCS). HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and F12 (1:1) supplemented with 10% FCS at 37°C in a 5% CO<sub>2</sub> incubator.

### **BrdU tracking and cell cycle progression:**

HeLa cells were cultured in the presence of 10 µM BrdU for 24 hrs. After removing BrdU from the media, cells were further incubated for 0, 20, or 40 hrs. After washing with PBS, cells were fixed in 70% ethanol at -20°C and washed again with PBS containing 0.5% FCS and 0.5% Tween-20. Following 2 M HCl treatment, cells were neutralized in 0.1 M sodium borate and stained with a fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody. Following brief washing with PBS, cells were stained with 50 µg/ml propidium iodide (PI) in PBS containing 10 µg/ml RNase

A. Samples were analyzed for DNA content (PI) on the x-axis and BrdU (FITC) on the y-axis by a FACScan flow cytometer (Becton Dickinson). Watson Pragmatic analysis was used to quantify cell cycle distribution of BrdU-incorporated cells [15].

#### **The ssDNA patch assay:**

Monolayer cells ( $1-3 \times 10^4$ ) seeded onto a coverslip (18 mm diameter) were grown for 30 hrs in culture medium containing 10  $\mu\text{g/ml}$  BrdU. After removing BrdU with fresh media, cells were treated with various DNA damaging agent for 1 hr. Cells were briefly washed with fresh media and further incubated for indicated period. For immunofluorescent analysis, cells were washed with PBS, fixed in ice-cold absolute methanol for 30 min at  $-20^\circ\text{C}$ , and rinsed in ice-cold acetone for few seconds. After washing four times with PBS, cells were incubated with an anti-BrdU monoclonal antibody in PBS containing 0.5% BSA for 2 hrs. After washing with PBS, samples were incubated with FITC-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) for 1 hr. Slides were mounted in 90% glycerol, 0.1 M Tris-HCl (pH 8.0), and 2.3% 1,4-diazobicyclo-2,2,2-octane, 4',6-diamidino-2-phenylindole (2 mg/ml). Images were collected using a Ziess LSM-510 confocal microscopy.

#### **Transfection of cells with siRNA:**

The siRNA specific for XPG and XPF, as well as a control-siRNA were prepared from Dharmacon Reserch Co. (Laffayette, CO). We also synthesized an antisense XPG-morpholino (25-mer; 5'-CCA GAG CCC CTG GAC CCC CAT GAG G-3'; underlined letters represent translation start site) (Gene Tools, LLC, Philomath, OR). HeLa cells ( $1-3 \times 10^4$ ) were plated on 6-well plates and incubated for 12-16 hrs prior to transfection. Cells were washed once with fresh medium and added siRNA (0.2-0.4  $\mu\text{M}$ ) diluted in DMEM/F12 to a final volume of 200  $\mu\text{l}$ . In a separate tube, 6  $\mu\text{l}$  of

Oligofectamine transfection reagent (Invitrogen, Carlsbad, CA) was mixed with 15  $\mu$ l of DMEM/F12 and incubated for 10 min at room temperature. Diluted siRNA samples were combined with the Oligofectamine mixtures and incubated for 20 min at room temperature before adding to cells. After incubation for 4 hrs, 0.5 ml culture medium containing 30% serum was added without removing the transfection mixture. Following incubation for 24-96 hrs, cell lysates were prepared and examined for efficacy of siRNA by western blot analysis.

#### **Preparation of cell lysates and immunoblot analysis:**

Cells ( $1 \times 10^5$ ) were collected, washed with PBS, and lysed in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM  $MgCl_2$ , 0.2 mM EDTA, 0.5% Triton X-100, 20 mM  $\beta$ -glycerolphosphate, 1 mM sodium vanadate, 1 mM DTT, protease inhibitor cocktails (Sigma Chemical Co.). Cell lysates (50  $\mu$ g) were loaded onto a SDS-PAGE and, following gel electrophoresis, proteins were transferred to a PVDF membrane (Millipore, Billerica, MA) and immunoblotted with primary antibody followed by peroxidase-coupled secondary antibody (Amersham, Piscataway, NJ) and an enhanced chemiluminescence (Amersham, Piscataway, NJ) reaction prior to visualization on Kodak-o-mat film.

#### **Association of XPG with chromatin following ICL damage:**

HeLa cells ( $1 \times 10^5$ ) were plated in a 100 mm culture dishes and transfected with pEGFP-XPG using a transfection reagent (FuGENE 6). After 24 hrs, cells were treated with MMC (20  $\mu$ M) for 1 hr and harvested at various time points for nuclei preparation. Isolated nuclei were washed with a buffer containing 0.3M NaCl, and chromatin-associated proteins were separated on 8% SDS-PAGE and analyzed for XPG by Western blot. Ku80 was used as a loading control. For immunofluorescence analysis of chromatin-associated XPG, HeLa cells were grown on a slide and transfected with

pEGFP-XPG using FuGENE 6. After 24 hrs, cells were treated with DNA damaging agent for 1 hr and further incubated for 2 hrs before cell harvest. Cells were treated with lysis buffer (25 mM Hepes, pH 7.5, 0.3 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 % Triton X-100, 0.5mM DTT, 1mM PMSF) for 10 min on ice, washed once with PBS, and fixed in ice-cold acetone-methanol (1:1) for 7min at -20°C. Slides were mounted in 90% glycerol, 0.1 M Tris-HCl (pH 8.0), and 2.3% 1,4-diazobicyclo-2,2,2-octane, 4',6-diamidino-2-phenylindole (2 mg/ml). Images were collected using a Ziess LSM-510 confocal microscopy.

## RESULTS

### **Mammalian cells treated with MMC induced formation of unique ssDNA foci**

MMC induces guanine-N<sup>2</sup> monoadducts or a DNA crosslink in the minor groove between two guanines at their 2-amino groups, which can be repaired through translesion bypass, recombinational repair, or nucleotide excision repair pathway [16,17,18]. Regardless of the types of DNA damage, initiation of MMC-induced DNA repair is likely to involve dual incisions of one strand of the damaged DNA site in mammalian cells, which results in the generation of ssDNA patch [2]. We therefore examined whether MMC treatment induces formation of the ssDNA foci in mammalian cells. To visualize the ssDNA patches, BrdU-labeled cells were treated with MMC and, the ssDNA patches were identified using an anti-BrdU antibody (BD Biosciences, San Jose, CA) that specifically recognizes BrdU on ssDNA not dsDNA (Figure 1A). DNA histogram analysis showed that intensity of BrdU labeling (y-axis) reduced at half every twenty hours (Figure 1B), suggesting that majority of cells were actively involved in cell cycle progression following BrdU incorporation. In the ssDNA foci assay, WT human fibroblast cells formed distinctive foci following MMC treatment (Figure 2A). These foci likely represented the ssDNA patches at the crosslink damage sites since the cells treated with methyl methane sulphonate (MMS) or adriamycin did not show such foci (Figure 2A). Furthermore, WT cells treated with both psoralen and ultraviolet light A (UV-A), but not either alone, also showed the ssDNA foci similar to those treated with MMC (Figure 2B), suggesting that MMC-induced ssDNA foci represent the ssDNA patches produced during crosslink repair *in vivo*.

### **Human mutant cells defective in XPE or XPG failed to form the ssDNA foci following MMC treatment**

Several XP proteins are involved in either recombination-dependent or –independent crosslink repair *in vitro* [17,19]. We examined WT cells and XP-deficient fibroblast cells for formation of the ssDNA foci following MMC treatment. WT cells and XPF-deficient cells showed distinctive ssDNA-specific patches following MMC treatment, whereas cells defective in XPE or XPG did not (Figure 3A), suggesting that MMC-induced ssDNA patch assay is different from those observed in NER pathway [20]. Similar results were obtained when cells were treated with both psoralen and UV-A (Figure 3B), suggesting that the ssDNA foci represent an essential step in the repair of crosslink damage. It should be pointed out that only 20-30% of WT and XPF-deficient cells were positive in forming the foci following MMC treatment (data not shown), suggesting that formation of the ssDNA foci may occur when cells are in S-phase of the cell cycle [7]. This is in keeping with a recent observation that crosslink damage induced double-strand break that forms during DNA replication [5].

#### **Lack of XPF significantly delayed MMC-induced ssDNA foci formation *in vivo***

Both XPF and XPG are structure-specific endonucleases that play essential role in nucleotide excision repair [2,6]. A positive role for XPF in the early stage of interstrand crosslink repair has been reported previously [2,5,11]. A question arises whether XPG and XPF play a unique role in formation of the ssDNA foci following MMC treatment. We therefore carried out kinetic analysis of the ssDNA foci formation with WT and XP-deficient cells following MMC treatment. WT cells showed the ssDNA foci within 3 hrs following MMC treatment (Figure 4). The foci formation reached maximum in 24 hrs and completely disappeared in 72 hrs, suggesting that the foci represent ssDNA patches occurred during the early stage of crosslink repair. Repair of MMC-induced damages occurred at a significantly slower rate than NER that usually completes within 2 hrs

following UV damage [21,22]. A mutant defective in XPG or XPE showed no foci formation up to 96 hrs following MMC treatment, while XPF mutant formed the ssDNA foci much later time point (24 hrs post-treatment) than WT cells (3 hrs post-treatment) (Figure 4), suggesting that XPF, in addition to XPE and XPG, is also involved in MMC-induced crosslink repair.

#### **A targeted inhibition of XPG abolished formation of the ssDNA foci following MMC treatment**

Human XPG mutant cells failed to form ssDNA patches following MMC treatment, suggesting that XPG may have a unique role in the crosslink repair. A possibility exists, however, that human XPG-deficient cells may have additional mutation(s) at other gene(s) crucial for inducing ssDNA patches following MMC treatment. To address this concern, WT human fibroblast cells were treated with siRNA specific to XPG or XPF, and examined for MMC-induced ssDNA foci formation. Both XPG- and XPF-siRNA were highly effective since the treatment of HeLa cells with either siRNA significantly lowered protein expression to an undetectable level (Figure 5A, lanes 3-6) under the conditions where a control siRNA showed little or no effect (Figure 5A, lane 2). A targeted inhibition of XPF also led to a significant decrease in ERCC-1, an XPF-associated protein (Figure 5B), suggesting that XPF stabilizes ERCC-1 by forming a complex [23]. Treatment of cells with XPG-siRNA completely abolished formation of the ssDNA foci, whereas XPF-siRNA showed little or no effect (Figure 5C), suggesting that XPG has a unique role in the repair of MMC-induced DNA damages that likely occurred during the early stage of crosslink repair.

#### **A targeted inhibition of XPG sensitizes HeLa cells in response to ICL damage**

Since siRNA effectively inhibited XPG expression and formation of the ssDNA foci in HeLa cells, we examined whether a targeted inhibition of XPG sensitizes cells following crosslink damage.



HeLa cells were treated with either control- or two different XPG-antisense RNAs (Morpholino A or B) for 72 hrs and plated in a 96-well plate ( $1 \times 10^4$  cells/ well). Unlike many antisense structural types (e.g. siRNA), Morpholinos do not degrade their target RNA molecules, instead, they sterically block binding to a target sequence within an RNA and simply getting in the way of molecules which might otherwise interact with the RNA. Treatment of cells with the target (XPG)-morpholino lowered the XPG expression to undetectable level (lanes 3 & 4, Figure 6) under the conditions where the control morpholino showed a little or no effect (lane 2, Figure 6), suggesting that the antisense morpholino strategy was effective in targeting XPG in cancer cells.

Cells were treated with various DNA damaging agents and incubated further at 37°C for 72 hrs. Cell treated with mock or a control antisense RNA was resistant up to 1  $\mu$ M of cisplatin (CDDP) treatment. On the other hand, cells treated with XPG-specific probe were sensitive to cisplatin treatment ( $IC_{50}=0.5 \mu$ M; Figure 6, panel A). Similar to cisplatin, cells also showed extreme sensitivity to MMC treatment only when treated with XPG-specific probe (Figure 6, panel B). In contrast, cells showed relatively mild sensitivity in response to alkylating agent (MMS) or adriamycin when pretreated with XPG-specific probe (Figure 6, panels C & D). This result also supports a positive role for XPG in the repair of crosslink damage.

#### **An association of XPG with chromatin was induced upon crosslink damage**

To further investigate a potential role for XPG in the repair of crosslink damage, nuclei were isolated from HeLa cells following treatment with MMC or cisplatin, and examined for association of XPG. Western blot analysis indicated that most of XPG was not associated with chromatin under normal conditions (Figure 7A, lane 1; 7B, first panel), whereas MMC treatment immediately induced the interaction of XPG with chromatin (Figure 7A, lanes 2-3). An immunofluorescence study with isolated nuclei from HeLa cells also showed an induced association of XPG with chromatin

following MMC or cisplatin treatment (Figure 7B). These observations support a positive role for XPG in perhaps an early stage of crosslink repair.

## DISCUSSION

MMC induces both intra- and interstrand DNA crosslinks that block DNA replication and transcription. It is not known how MMC-induced DNA damages are repaired in mammals, however, initiation process likely involves dual incision on the first strand and generates ssDNA patch on one strand, while damaged DNA piece hanging on the second strand [2,11,23]. Filling-in the gap of first strand DNA can be accomplished by either translesion bypass or recombinational repair mechanism [18]. In this study, we described an *in vivo* analysis of MMC damage and its repair using an assay that detects the ssDNA patches produced during the crosslink repair. We found that MMC-induced formation of the ssDNA patches was completely dependent on the presence of XPG and XPE, suggesting that these two proteins may have unique role(s) in the early stage of crosslink repair.

Both XPF and XPG are structure-specific endonucleases essential for the release of an oligonucleotide containing the lesion in the early stage of NER [6]. Following MMC treatment, however, XPG (3'-endonuclease) was crucial for formation of the ssDNA foci, whereas XPF (5'-endonuclease) affects kinetics of the foci formation (Figure 4). Although detailed mechanism of MMC-induced ssDNA foci is yet to be defined, our *in vivo* finding was somewhat contradictory to the previous *in vitro* observations that excinuclease complex involving XPF-ERCC1 was responsible for the recognition of crosslink damage and generation of dual incisions [2,5,17,19]. This discrepancy could be explained by the fact that the *in vivo* crosslink repair may be complex and involve more than one repair pathway [18], whereas the previous *in vitro* studies were reconstituted predominantly by one ICL repair pathway. For example, MMC-induced ssDNA patches may be due to an introduction of single-strand nicks that eventually permit ssDNA foci formation. Alternatively, XPG and XPF-ERCC1, unlike NER, may have separate role(s) in forming the ssDNA patches that

represent an early stage of crosslink repair pathway, whilst both participates in the later stage of ICL repair such as incision of the second strand.

It is intriguing that MMC-induced ssDNA foci formation in XPF mutant cells occurred much later than that observed in WT cells (Figure 4). ERCC1-XPF, through its role in nucleotide excision repair (NER), is essential for the repair of crosslink DNA damage. ERCC1-XPF is also involved in recombinational repair processes distinct from NER. Earlier genetic data implicate that the XPF-ERCC1 was required for crosslink repair via homologous recombination-mediated double-strand break (DSB) repair pathway [5]. However, an increased amount of DSB and the phosphorylation of histone variant H2AX (gamma-H2AX) were observed in both WT and *Ercc1*(-/-) cells following MMC treatment, although in *Ercc1*(-/-) cells MMC-induced gamma-H2AX foci persisted at least 48 hrs longer than in WT cells [5], suggesting that XPF-ERCC1 is required for the resolution of MMC-induced DSBs, although DSBs occur after crosslink damage via an ERCC1- (or XPF-) independent mechanism [5]. It would be interesting to see whether the MMC-induced ssDNA foci actually represent the early stage of crosslink repair via homologous recombination-mediated DSB repair pathway.

We do not know what specific role XPG plays in forming the ssDNA foci following MMC treatment *in vivo*. XPG is not only essential for dual incisions at the crosslink lesion, but is also required in a non-enzymatic capacity for occurrence of the second, 5'-incision by the ERCC1-XPF heterodimer, another structure-specific endonuclease of opposite polarity [2,24,25]. XPG interacts with a number of repair factors involved in NER/transcription-coupled repair (TCR) [transcription factor II-H (TFII-H), replication protein A (RPA), and proliferating cell nuclear antigen (PCNA)] [26,27,28,29]. XPG is also involved in base excision repair of oxidative DNA damages [30,31]. Our findings

described here show that XPG was exclusively localized in the nucleus and its interaction with chromatin was induced following MMC treatment (Figure 7). Furthermore, a targeted inhibition of XPG significantly increased cell sensitivity in response to crosslinking agents (Figure 6), suggesting that XPG is also involved in ICL repair pathways. XPG and its endonuclease activity may directly be involved in generation of the ssDNA patches following MMC damages, however, a possibility exists that a unique role for XPG in crosslink repair may be through mediating physical interaction with other repair factors. MMC-induced formation of the ssDNA patches was also dependent on the presence of XPE (Figure 3). XPE was originally identified as a damage-specific DNA-binding protein (32) and may be involved in recognizing MMC-induced DNA damage, although we do not know what specific role it plays in the repair of MMC-induced DNA damage.

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## FIGURE LEGENDS

**Figure 1. Schematic diagram of the ssDNA foci assay. Panel A.** This assay is designed to evaluate the ICL repair activity in mammalian cells. 5'-bromo-2'-deoxyuridine (BrdU)-incorporated fibroblast cells were treated with DNA crosslinker (MMC) for 1 hr and further incubated in fresh media for the repair of damaged DNA. An anti-BrdU antibody recognized BrdU on ssDNA patches and exhibited distinctive nuclear foci. An antibody was highly specific for BrdU on ssDNA and did not show any interaction with BrdU on dsDNA (data not shown). **Panel B.** HeLa cells were monitored for cell cycle progression following labeling with BrdU. After the BrdU labeling (10  $\mu$ M) for 24 hrs, cells were harvested at various time points, washed once in PBS, and fixed in 70% ethanol at -20°C. The cells were washed with PBS containing 0.5% FCS and 0.5% tween-20, denatured in 2M HCl, neutralized in 0.1 M sodium borate, and stained with an anti BrdU antibody conjugated to FITC. The cells were washed in PBS as above and stained with 50  $\mu$ g/ml PI in PBS containing 10  $\mu$ g/ml RnaseA. The samples were analyzed by bivariate FACS analysis, with DNA content (PI) on the x-axis and BrdU (FITC) on the y-axis. The dot plots and the histograms representing the cells are shown.

**Figure 2. Formation of the ssDNA foci was observed following treatment with MMC, not with adriamycin or MMS. Panel A.** Following BrdU labeling of cells for 30 hrs, WT human fibroblast cells were treated with various DNA damaging agents (2  $\mu$ M MMC, 100  $\mu$ M MMS, or 10  $\mu$ M adriamycin) for 1 hr. Cells were further incubated in fresh media for 24 hrs prior to cell fixing and immunostaining with an anti-BrdU antibody specific for ssDNA patches. Fluorescence images were analyzed by confocal microscope. **Panel B.** WT cells were treated with 8-MOP (100  $\mu$ M), UV-A (500 J/m<sup>2</sup>), or both

8-MOP + UV-A prior to immunostaining analysis using an anti-BrdU antibody specific for ssDNA patches.

**Figure 3. Formation of the ssDNA foci following crosslink damage in WT cells and various XP-mutant cells.** **Panel A.** WT cells or various XP-mutant cells were treated with either 0 (top panels) or 20  $\mu$ M (bottom panels) MMC for 1 hr, and further incubated for 24 hrs prior to immunofluorescence with an anti-BrdU antibody specific for ssDNA patches. **Panel B.** WT or XP-mutant cells were mock-treated (first row), 100  $\mu$ M 8-MOP (second row), 500 J/m<sup>2</sup> UV-A (third row), or 100  $\mu$ M 8-MOP + 500 J/m<sup>2</sup> UV-A (fourth row), and further incubated for 12 hrs prior to immunofluorescence as described in panel A.

**Figure 4. Kinetic analysis of the ssDNA foci formation following MMC treatment.** WT and various XP-mutant human fibroblast cells were examined for formation of the ssDNA foci following MMC treatment. Cells were treated with 2  $\mu$ M of MMC for 1 hr prior to cell harvest at various time points and measured for the ssDNA foci-positive cells per coverslip (18 mm diameter) in a dish. Numbers were the mean values from four separate samples.

**Figure 5. A targeted inhibition of XPG abolished formation of the ssDNA foci following MMC treatment.** **Panel A.** Effect of XPG- or XPF-specific siRNA on protein expression. WT human fibroblast cells were either untreated (lane 1; upper & lower panels), or treated with a control (scrambled) siRNA (lane 2; upper & lower panels), XPF-specific siRNA (lanes 3-6; upper panel), or XPG-specific siRNA (lanes 3-6; lower panel). After harvesting cells at various times, expression of XPG or XPF was analyzed by immunoblot. Expression of Ku80 was included as an internal control for individual lanes. **Panel B.** A targeted inhibition of XPF also affected expression of its binding

partner, ERCC-1. WT human fibroblast cells were either untreated (lane 1), or treated with a control (scrambled) siRNA (lane 2), or XPF-specific siRNA (lane 3) for 48 hrs prior to harvest. Cell extracts were prepared and analyzed for XPF and ERCC1 by Western blot. Expression of Ku70/Ku80 was included as an internal control (bottom panel). **Panel C.** A siRNA targeting of XPG not XPF abolished formation of the ssDNA foci following MMC treatment. WT human fibroblast cells were treated with indicated siRNA for 24 hrs prior to treatment with 20  $\mu$ M MMC for 1 hr. Following incubation for 24 hrs, the ssDNA foci were visualized using immunofluorescence with an anti-BrdU antibody specific for ssDNA patches. Cells were analyzed by confocal microscope.

**Figure 6. Effect of a targeted inhibition of XPG on cell sensitivity following treatment with various DNA damaging agents.** HeLa cells were incubated with either control- or two different XPG-specific antisense RNA for 48 hrs prior to treatment with DNA damaging agent. Cells were treated with various concentrations of cisplatin (panel A), MMC (panel B), MMS (panel C), or adriamycin (panel D) for 72 hrs before cell survival assay. Percentage of cell survival (%) was obtained from MTT assay at the indicated concentration and the results were the averages of three assays.

**Figure 7. Chromatin association of XPG was induced upon MMC damages. Panel A.** Western blot analysis of chromatin-associated XPG following MMC damage. HeLa cells were treated with MMC (20  $\mu$ M) for 1 hr and harvested at various time points. Isolated nuclei were washed with a buffer containing 0.3M NaCl, and chromatin-associated proteins were separated on 8% SDS-PAGE and analyzed for XPG by Western blot. Ku80 was used for Western analysis as a loading control. **Panel B.** Immunofluorescence analysis of chromatin-associated XPG following treatment with

either MMC or cisplatin. HeLa cells were grown on slides and treated with nothing (first row), 20  $\mu$ M MMC (middle row), and 50  $\mu$ M cisplatin (third row) for 1 hr and incubated for 2 hrs prior to chromatin preparation. Images were collected using a Ziess LSM-510 confocal microscopy.

Figure 1A

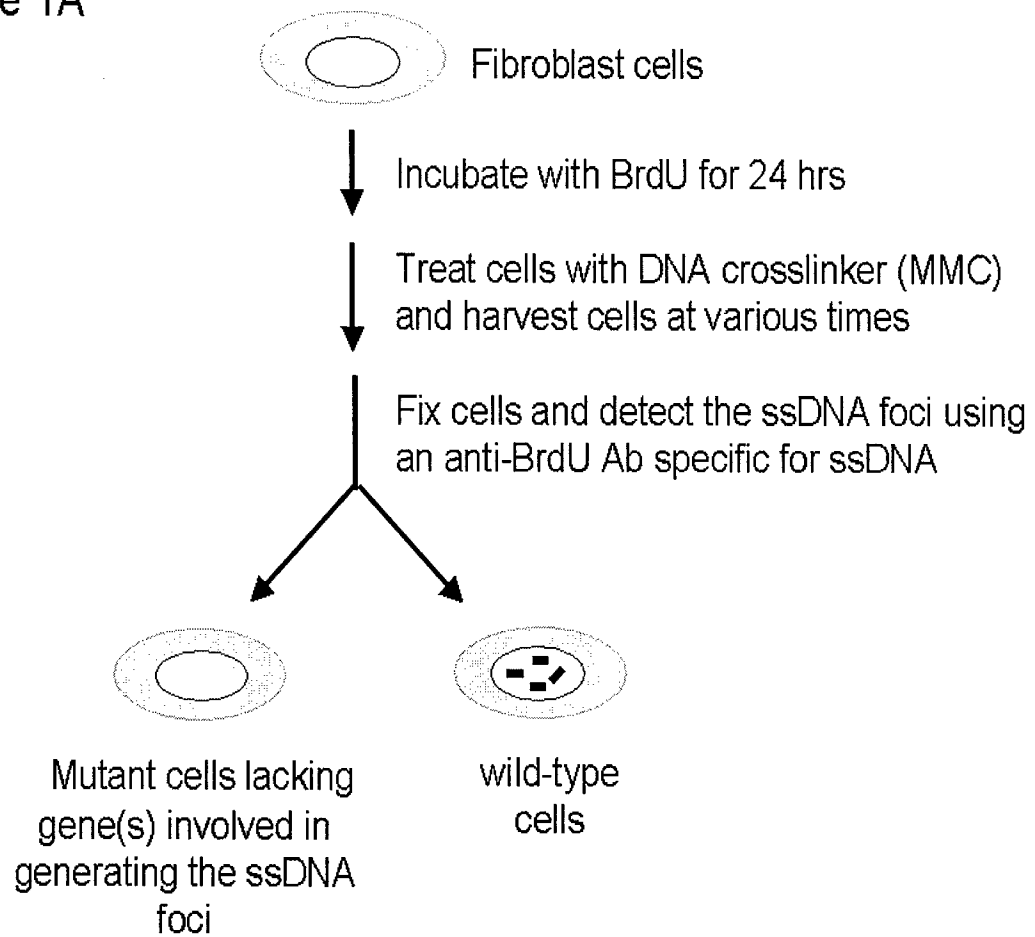


Figure 1B

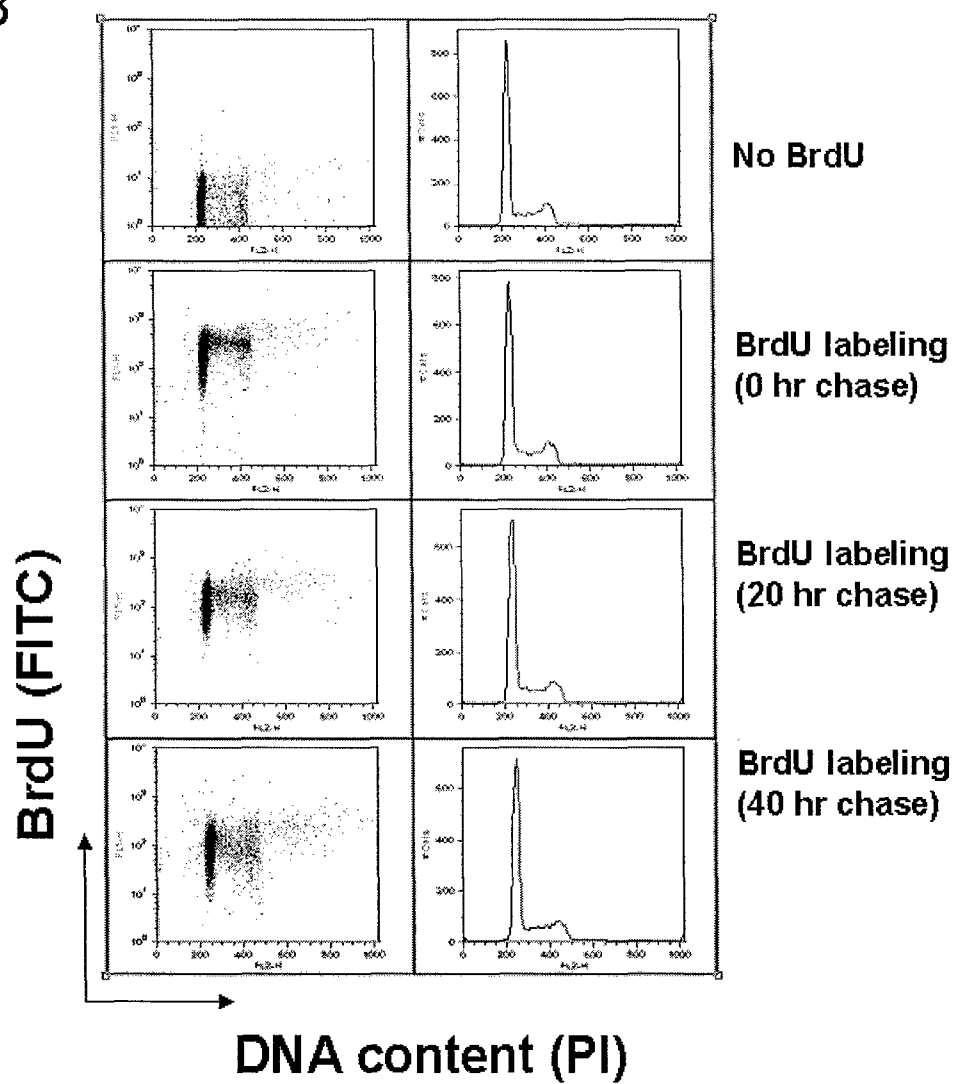
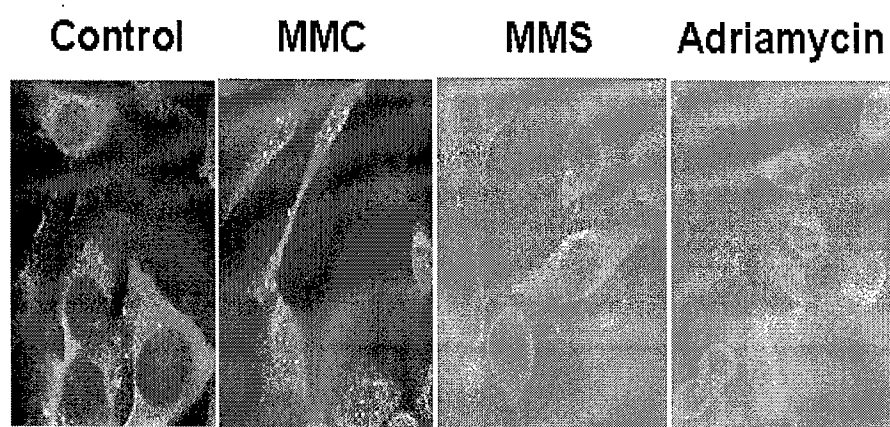




Figure 2

A.



B.

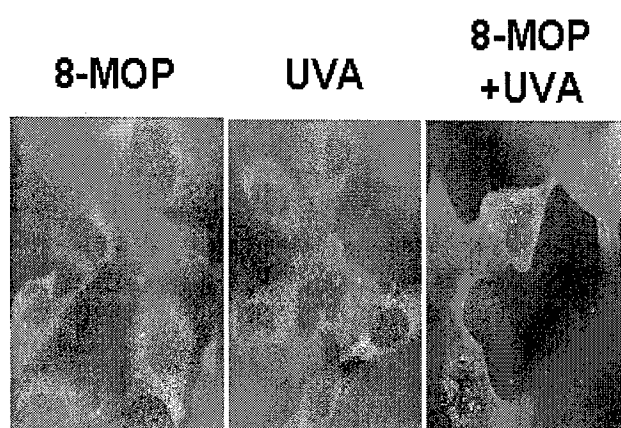


Figure 3A

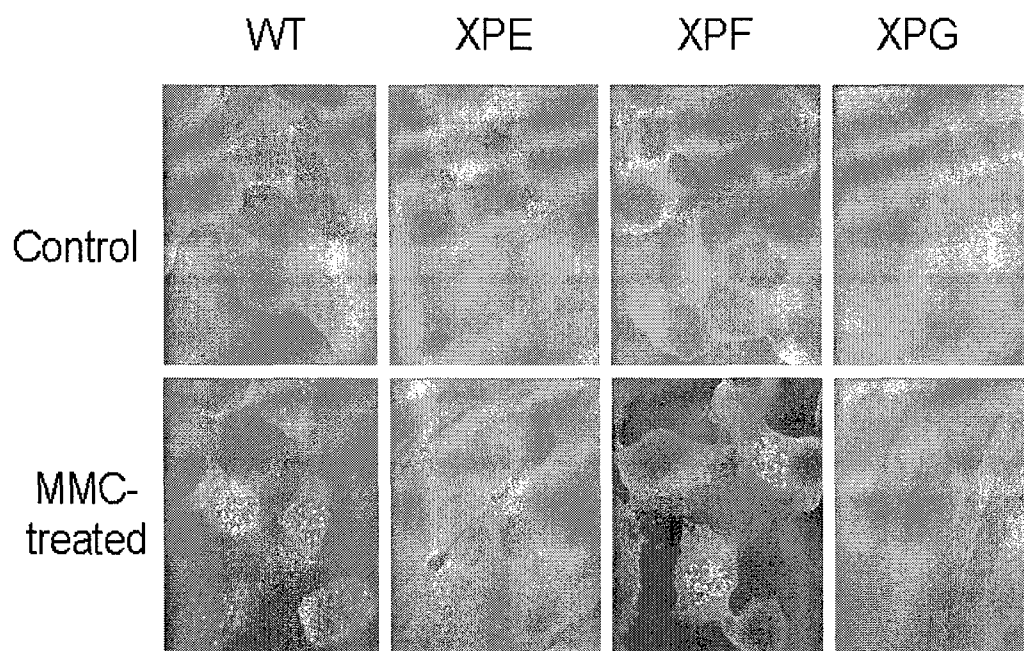


Figure 3B

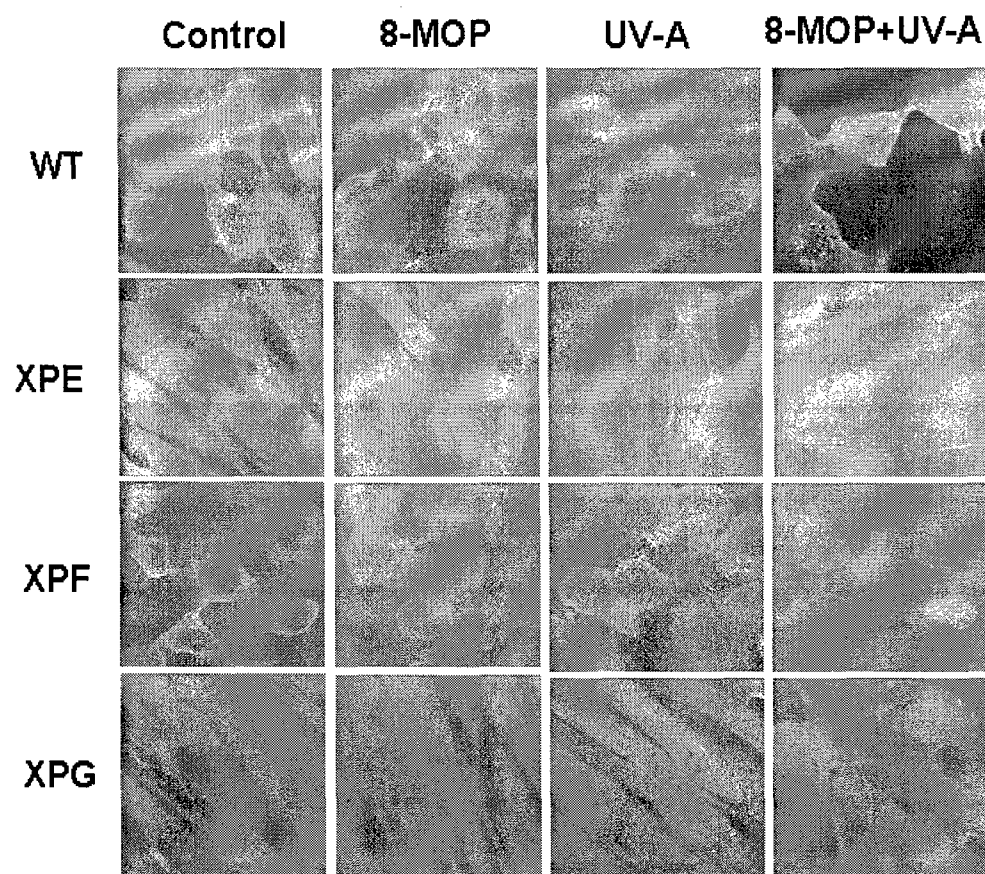


Figure 4

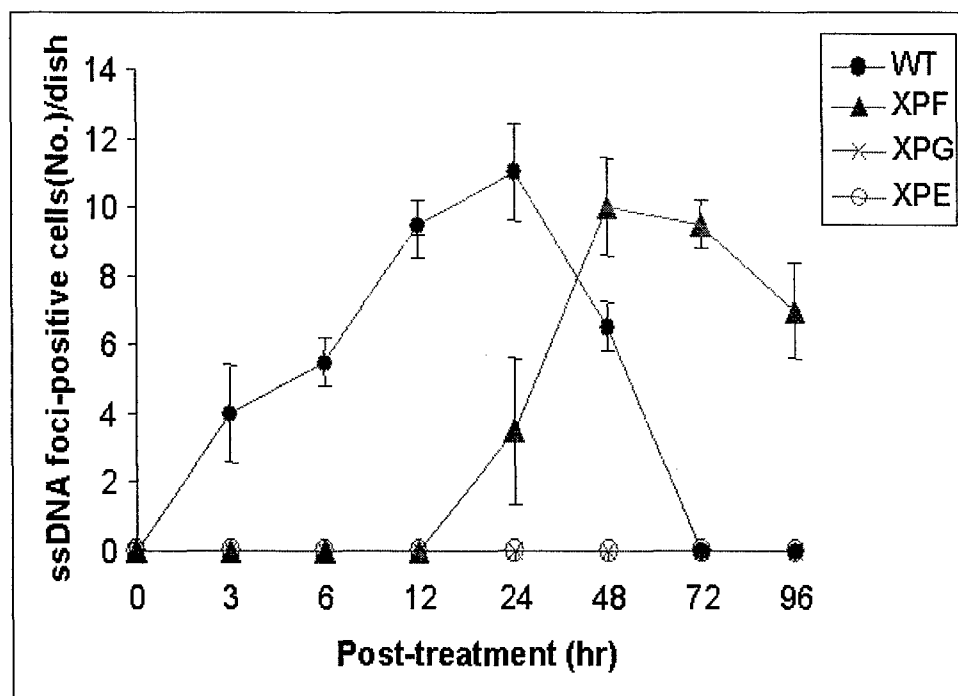


Figure 5A

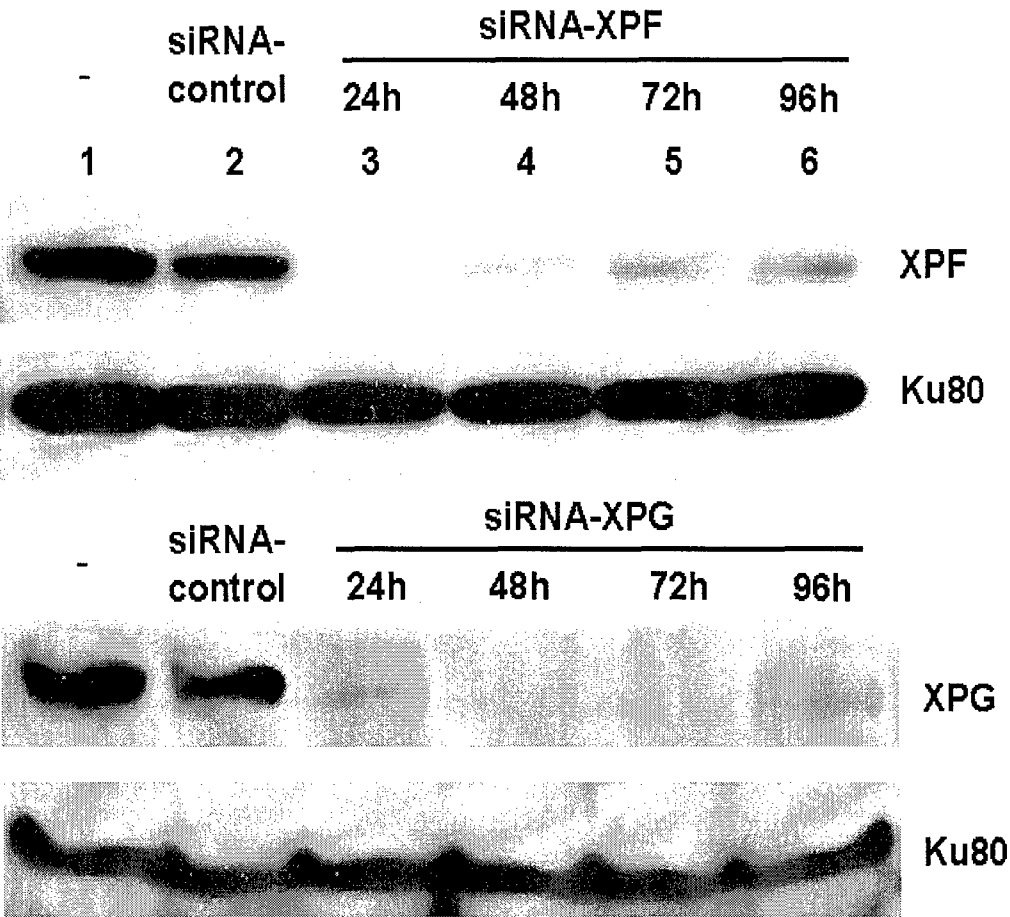


Figure 5B

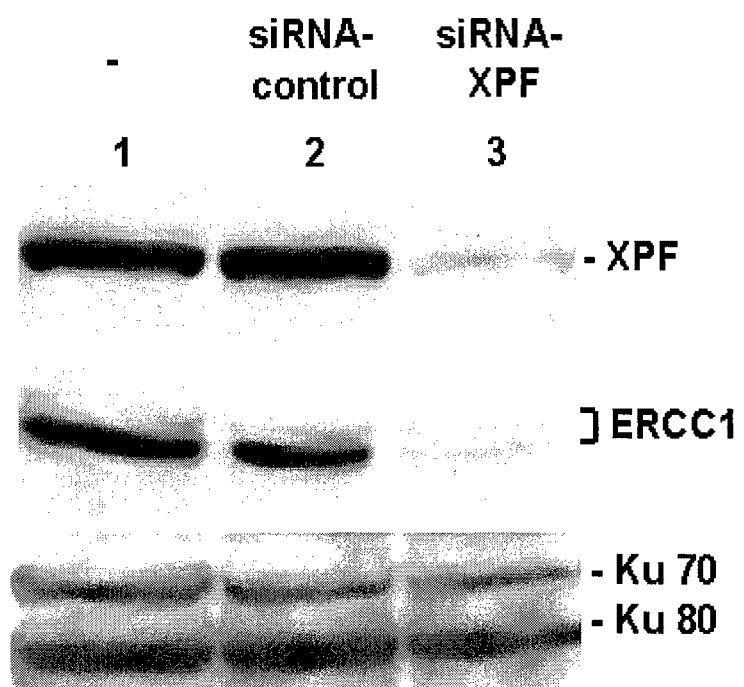


Figure 5C

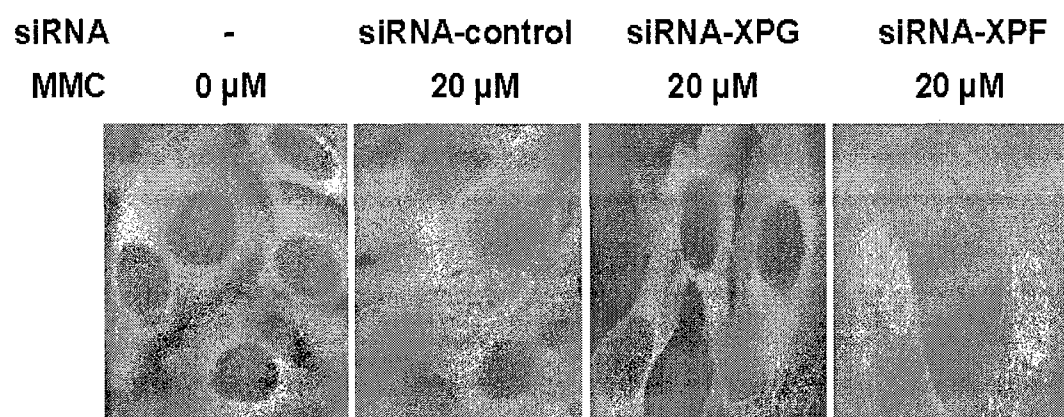


Figure 6

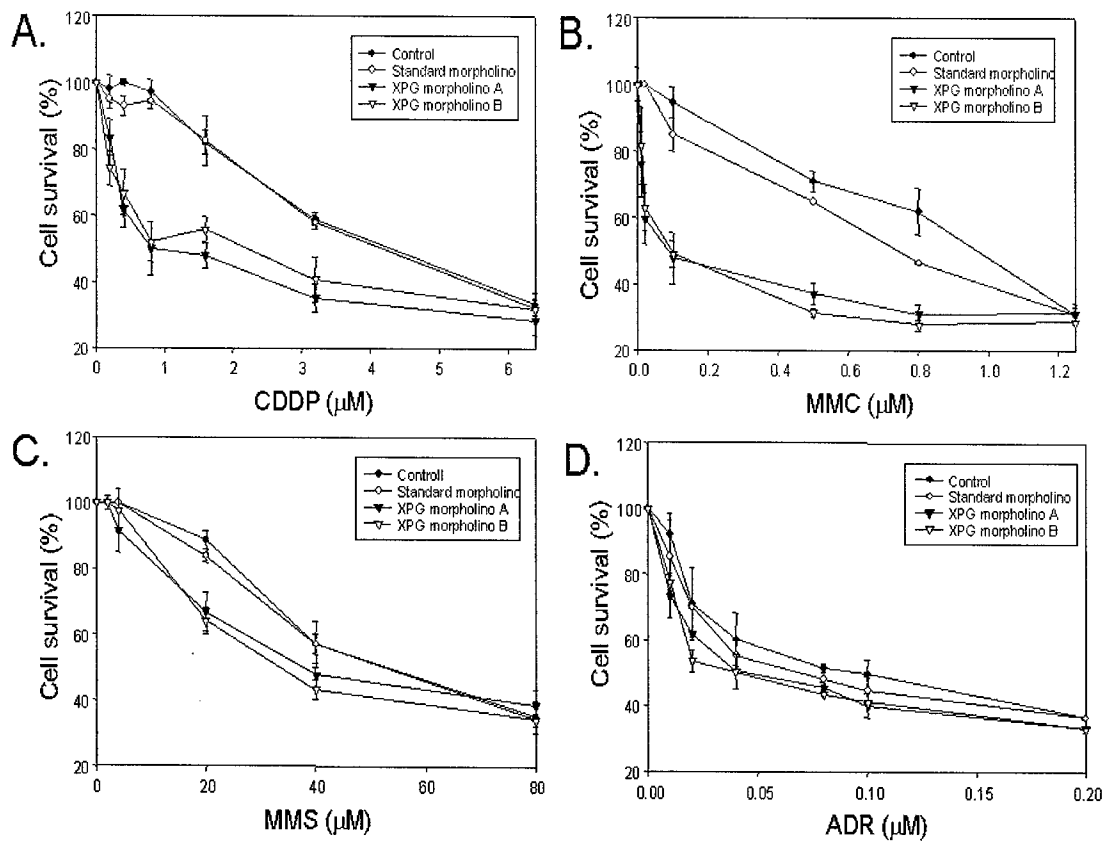




Figure 7

